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SOME OBSERVATIONS ON A LUMINESCENT FRESHWATER LIMPET FROM NEW ZEALAND

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In view of the statement of Harvey (1940) that ". . . luminous animals are practically entirely marine or terrestrial. No examples of freshwater luminous organisms are known except bacteria and an aquatic glowworm . . .," the luminous properties of the New Zealand freshwater mollusc, *Latia neritoides,* would appear to be a unique and surprising phenomenon. This, together with the fact that, apart from the cephalopods, only three luminescent species of molluscs have been hitherto recorded (Harvey, 1940) is sufficient justification for a full investigation of the apparently exceptional properties of this freshwater limpet.

Latia is a monotypic genus restricted to the North Island of New Zealand. It belongs to the family Ancylidae in which no other luminescent forms have been recorded. The two other New Zealand species (*Gundlachia neozelanica* and *G. lucasi*) are certainly not luminescent.

The luminescence of Latia was first recorded by Suter in 1890 and again referred to in his comprehensive Manual of New Zealand Mollusca (1913). Although the phenomenon is well known to many New Zealanders, its nature and mechanism have never been studied.

It was with this end in view, therefore, particularly in the light of modern researches in the field of bioluminescence, that the present investigation has been undertaken.

Material and Methods

Specimens of *Latia neritoides* were collected near Auckland in December, 1949, and were immediately placed in Bouin's fluid. Subsequently during the investigation, living specimens were obtained in May, 1950, for experimental and histological purposes. It was found that these animals remained alive in the laboratory for more than three weeks if placed in brown glass bottles in the dark, whereas if kept in white glass bottles and exposed to the light they died within a day or two.

In order to contrast the histological structure of Latia with that of an allied non-luminescent form, specimens of *Gundlachia lucasi* were collected locally and submitted to simliar histological procedures.

Luciferin-luciferase reaction. Three animals were shaken in 10 ml. of water in a test-tube at room temperature for 10 minutes. The solution was then de-

canted into another test-tube and allowed to stand until all luminescence had disappeared, leaving a solution of the thermolabile luciferase. Three more animals were placed in another test-tube with 10 ml. of water at 70° C., crushed, kept at that temperature in a water bath for 10 minutes and then allowed to cool. At 70° C. the thermolabile luciferase is denatured and the substrate, luciferin, is thus obtained unoxidised in solution.

Five ml. of clear solution were then decanted from each tube, neither of which at this stage showed any luminescence. Upon mixing the two samples, however, a brilliant pale green light was produced. This indicated a positive luciferin-luciferase reaction.

The possibility that the presence of "cytolytic" substances in the hot-water solution might cause luminescence, by breaking down residual granules of luciferin in the cold-water extract, was disproved by the method described by Harvey (1940) of adding to the cold water solution several drops of ether which produces a similar effect. No luminescence resulted in this solution, thus proving the absence of residual granules of luciferin, while the presence of the ether did not inhibit the production of light when the two solutions were mixed. This appeared to confirm the conclusion that the reaction is a true luciferin-luciferase oxidation.

Histology. For histological study the animals were fixed in Bouin's fluid. They were embedded in paraffin and sectioned at 10 μ, both longitudinally and transversely. A variety of staining procedures were used: haematoxylin and eosin, Mayer's mucicarmine, and the Azan stain. An attempt was also made to study the innervation of the specific cells by means of the methylene blue technique, but this was unsuccessful and consideration of this aspect of the problem is deferred.

In order to determine the actual source of the luminescent material, several specimens were submitted to prolonged stimulation, by rubbing with a seeker the luminescent zones, in order to exhaust the specific cells. These animals were then submitted to the same histological procedures as before (Figs. 4, 5).

Observations

General. Latia is common in clear streams, often in rapids and other situations where the current is swift; living on the sides or undersurfaces of clean boulders, its limpet shape offers little resistance to the smooth flow of water. It is also found, but much less commonly, in lakes in places where there are clean rocks and considerable water movement, usually very close to the shore. An analysis of the mixed supply of water from the Waitakere Ranges near Auckland indicates its low salt content: sodium chloride was present as 3.04 parts and magnesium chloride as 0.76 parts per 100,000. Latia is abundant in all of these streams and from one of them the specimens used in this investigation were collected.

Like most other luminous forms, Latia shuns strong light and, as already stated, it soon dies if kept exposed to light. It has a well developed eye with the "pupil" directed forwards, and appears to be well adapted to perceive the direction of incident light.

Latia commonly reaches a length of 8.5 mm., a breadth of 6 mm. and a height of 3 mm. Its shell has a thin calcareous layer covered by a stout dark brown periostracum, smooth except for growth lines. Posteriorly, there projects forwards (horizontally within the shell) a semicircular shelf or lamella which on the right

side is prolonged forwards into a free calcareous lingula (Fig. 2) which in the living state supports the medial wall of the pneumostome (the inferior pallial lobe) (Fig. 1). Both the lamella and the lingula give origin to the muscular mass of the foot (Fig. 3) and so fix the shell very firmly to the body. Although probably a great advantage in a swift stream, this nevertheless greatly restricts the mobility of the animal relative to the shell, for when placed on its back it is unable to right itself; Gundlachia, which lacks these processes, is able to perform the manoeuvre easily. The mantle cavity of Latia, like that of some marine Basommatophora (the Siphonariidae, the Gadiniidae), is water filled, but as in the Gadiniidae does not contain a branchia. The skeletal support afforded by the lingula appears to prevent both the closure and the over-dilatation of the pneumostome while directing a stream of water into the mantle cavity to flush it.

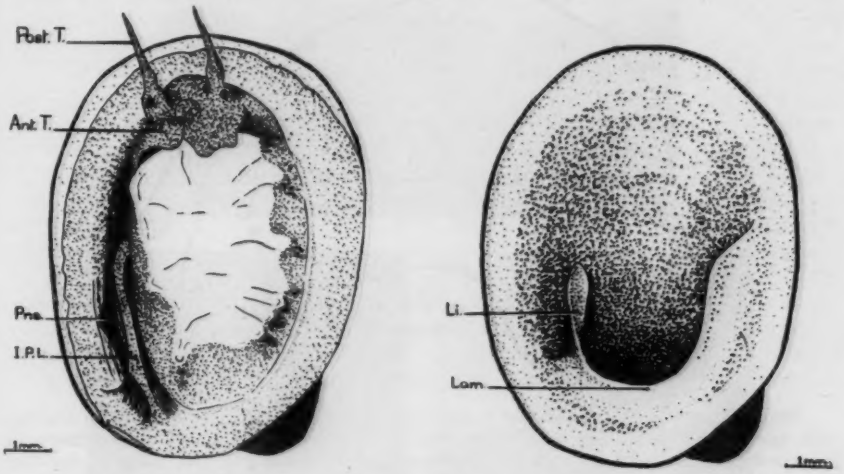


FIGURE 1. *Latia neritoides* Gray. Ventral view of the intact animal. All the parts of the animal that are shown are light producing, except the sole of the foot, the posterior tentacles, and the interior of the pulmonary cavity. Pns., pneumostome. I.P.L., inferior pallial lobe. Ant. T., anterior tentacle, Post. T., posterior tentacle.

FIGURE 2. The interior of the shell showing the semicircular lamella (Lam.) and the lingula (Li.)

Reference may be made to some remarks on the anatomy of Latia by Hutton (1881). Probably because of poorly preserved material he stated that the tentacles were transversely ringed and that the eyes were lateral to the bases of the tentacles. This is obviously not the case either in fresh or fixed specimens (Fig. 1). Hutton also gave the radular formula as $27 + 1 + 27$ but this is certainly wrong, since the number of laterals is approximately 110.

Latia, like the rest of its sub-order, is a vegetarian. The gut is usually filled with diatoms and what appear to be the remains of algae. Similar diatoms are found on the surface of the shell.

The luminescence. Whenever the animal is disturbed, as by shaking in a vessel or tapping the shell, luminous mucus appears in all parts of the groove between the foot and the mantle. This is visible even in diffused daylight as a greenish glow, and in the dark the effect is much more striking. The animals have never been seen to luminesce spontaneously at night either in the laboratory or in their natural habitat, but when they are stimulated they produce the glow equally well during the day or night. Where the mantle groove is touched with a seeker, that region in particular glows most strongly. Shaking the animal in a test tube or rubbing it, foot down, across a glass slide produces trails of glowing mucus from which the light slowly fades.

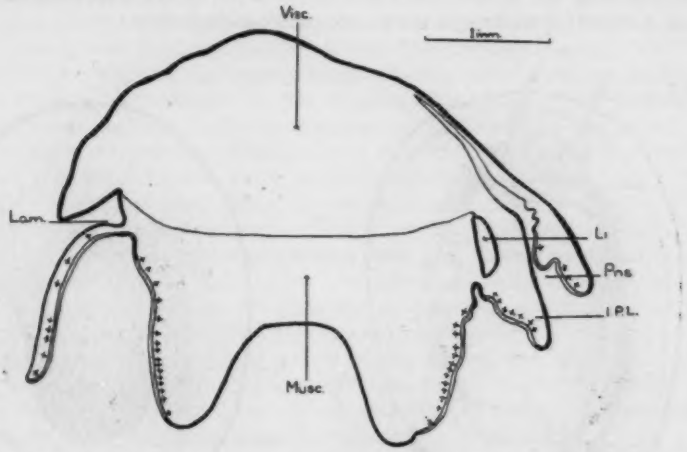
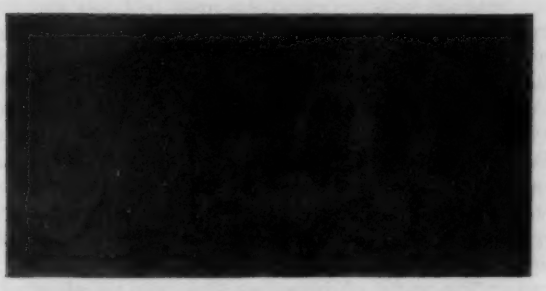
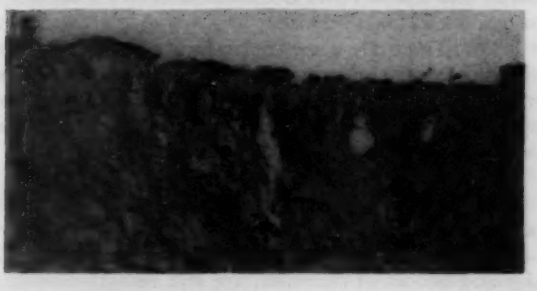


FIGURE 3. Outline of a transverse section (camera lucida). The crosses show the approximate number and distribution of granular cells. (These represent the average distribution in six non-consecutive sections from the same specimen.) Musc., muscular mass of the foot. Visc., viscral hump. (Other letters as for Figs. 1 and 2.)

Clearly then the phenomenon is an extracellular one, and experiments to determine the nature of the luminescence show that it depends on the action of luciferase upon the substrate luciferin, which appear to be produced together from specific areas, particularly along the walls of the mantle groove.

Histology. Examination of the sectioned animal shows that certain parts of its surface present a very striking and characteristic appearance. These regions are the surface of the head, the anterior tentacles, the lateral surfaces of the foot, the inferior pallial lobe, and the free surface of the mantle (but not of the pulmonary cavity). In all these situations, just beneath the simple cuboidal surface epithelium and lying in the loose subepithelial connective tissue, are large numbers of intermingled mucous cells and granular cells, together with branching melanophores and muscle fibres (Figs. 4, 6, 7). Since these regions correspond precisely to those from which the maximum luminous response may be obtained experimentally, they obviously represent the structural apparatus responsible for the luminescence.





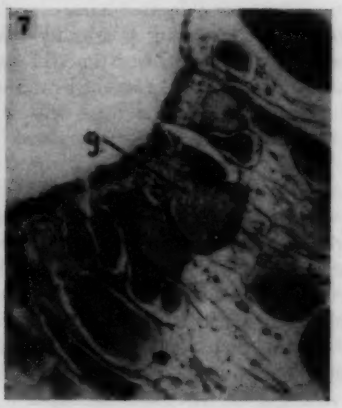


FIGURE 4. General view of luminous epithelium (Haematoxylin and Eosin) × 180.

FIGURE 5. A similar view after the animal had been stimulated to exhaustion (Haematoxylin and Eosin).

FIGURE 6. Two mucous cells in this figure show basally placed nuclei and the large size of the vacuole (Haematoxylin and Eosin) × 250.

FIGURE 7. The granular cell (g) in the center of the figure shows a flattened, basally situated, darkly stained nucleus and granules packing the whole cytoplasm (Azan).

The mucous and granular cells appear to be most probably the specific elements concerned in the phenomenon, since they alone are absent from the non-luminous areas, and they are not found in other non-luminous mollusca including the related Gundlachia.

1. Mucous cells. These are large elongated ovoid cells which may reach a length of 200 μ and a diameter of 40 μ. They taper markedly as they approach the surface epithelium and in many cases their secretion could be observed being extruded at the surface from the apex of the cell. The cells present large swollen vacuoles containing mucus, which stains pink or pinkish-purple with haematoxylin and eosin, purple with Azan, but is not stained by Mayer's mucicarmine. The cytoplasm, which is greatly attenuated, stains blue with Azan but is indistinctly stained with eosin. The nuclei of these distended cells are always basally situated and somewhat compressed.

2. Granular cells. These are elongated cells, rather smaller than the mucous cells (150 $\mu \times 30\,\mu$) but like them project into the epidermis. Their cytoplasm is packed with densely basophilic granules which in haematoxylin preparations generally obscure the nucleus. Where it is visible the nucleus is generally, but not always, basally situated and very flattened. With Azan the nuclei stain red, the granules blue.

3. Changes after stimulation. Instead of the large and distinctive mucous and granular cells, which were previously such a striking feature of the subepithelial tissue, it is now difficult to identify any specfiic cells at all. While empty mucous cells, now slender and devoid of secretion, are still recognisable here and there, it is practically impossible to identify with certainty the previously granular cells. There is, in fact, a strong possibility that not only are the entire contents of the granular cells discharged but that the nucleus is extruded as well, in which case mucous cells alone would remain in the exhausted luminescent areas. Whether or not this applies to all the granular cells, it is certainly the case that smears of mucus do contain nuclei of an appearance closely similar to those of the granular cells.

Discussion

The above observations establish quite clearly that Latia is a completely aquatic gasteropod with marked adaptations to life in strong currents; and that its bright luminescence is extracellular and is due to the secretion of luciferin and luciferase.

Harvey (1940) has stated that in only five of the very numerous orders of plants and animals in which luminescence occurs, can the luciferin-luciferase reaction be demonstrated *in vitro*. These include beetles (fireflies), some ostracods, a few polychaete worms, one squid, and now Latia joins *Pholas dactylus* as the second example in the lower mollusca (Plocamopherus and Phyllirrhoë have not yet been tested). Since the only other known example of fresh water luminous animals are the aquatic glowworms, it is interesting to compare Latia with these animals. These glowworms are actually larval coleoptera and have been recorded only from Asia. Annandale (1900) has described one (? Luciola sp.) from Calcutta; another (identified provisionally as *Pyrophanes similis*) was found on the Island of Celebes and described by Blair (1927); while two others from Japan (*Luciola cruciata* and *L. lateralis*) have been described by Okada (1928). Since in these forms, which all possess tracheal gills, the tracheae are full of air, the functioning of the luminescent organs is presumably the same as that in the adults which

are terrestrial fireflies. In these, in striking contrast to Latia, the production of light is intracellular and the luminous organs are covered like the rest of the body by a chitinous cuticle (Buck, 1948; Okada, 1928).

The luminous apparatus of Latia is similar to that of certain other luminous invertebrates (Dahlgren, 1915–17), particularly those with a naked epidermis or with only a thin cuticle, such as the coelenterates, polychaetes (especially Chaetopterus), and enteropneusta (Ptychodera). It bears, however, by far the closest resemblance to the luminous apparatus of another mollusc—the peculiar, pelagic opisthobranch *Phyllirrhoë bucephala,* in which both the mucous and the granular cells are almost identical with those of Latia; although I have not observed in Latia the prominent nerve-endings which are described by Dahlgren (1916a) in relation with the granular cells of Phyllirrhoë. In view, however, of the doubt cast on the numerous (and extremely conflicting) descriptions of light-cell innervation in the fireflies (Buck, 1948), the problem of whether or not there is a direct innervation of these cells in mollusca must remain open until such nervous connections have been demonstrated by recognised neurological techniques.

The possibility mentioned above, that the secretion of the granular cells in Latia may be holocrine, seems to be supported by some statements of Dahlgren. He suggests, for instance, that in Chaetopterus the granular cells may be "of a secretory type that are destroyed by one cycle of secretory activity" (1916b); again, when dealing with Pholas (1916a), he recalls Forster's observation that the granules in the granular cells of Pholas fill the cell and appear to be in contact with the nucleus. He comments that this is unusual except in cells which die on discharge of their contents but the mucus does not seem to have been examined for nuclei.

We see, therefore, that although Latia is quite unique among freshwater animals, the actual mechanism of its light-production closely resembles that of certain other marine luminous species, and thus does not bring us any closer to the solution of the very interesting problem: why is it that luminous forms, so common in the sea are so rare in fresh water? It is, however, possible to say now that the reason for their rarity cannot simply depend on a low concentration of salt.

Of the functions which may be attributed to the luminescence of Latia, no positive conclusions can be drawn. It has been suggested that luminescence might subserve three principal purposes not necessarily mutually exclusive: a lure for food; a sexual recognition signal; or a defensive mechanism. In the case of Latia, the first may be dismissed because the animal is a typical herbivore. The second, although it cannot be completely discounted, is unlikely for two reasons: Latia where it occurs is a common species, it is moreover an hermaphrodite and it would seem to have fewer rather than more difficulties to overcome than the nonluminous operculates which occur with it; furthermore its light production is not seasonal and is readily elicited by harmful stimuli.

As for the third possibility: what little evidence there is on the food habits of the fresh water fauna of New Zealand indicates that Latia is eaten by both trout (Phillips, 1929) and eels (Cairns, 1944) but detailed statistics are not available. It seems reasonable to conclude, however, that it is not unpalatable so that the luminescence cannot be equivalent to a warning coloration. A more obscure defensive action has been suggested for other luminous forms by Burkenroad (1943) who has put forward the view that light from specimens attacked by predators might attract secondary predators for the purpose of driving off the primary ones. How-

ever the problem is obviously one which cannot be solved until more detailed field work can be undertaken.

In conclusion, it may be stated that the luminescence cannot be regarded as physiological accident or metabolic by-product. The large size and high degree of specialization of the specific cells are themselves sufficient evidence to the contrary. Latia, of course, is not exceptional in lacking an obvious use for its light, for luminous species in which a function is known are the exception rather than the rule (Harvey, 1948).

Acknowledgments

I wish to express my gratitude to Professor W. E. Adams for allowing me to use the laboratories of this Department and for criticising the manuscript; to Mr. A. D. Mead, City Waterworks Engineer, Auckland, for providing the water analysis figures; and to my parents for collecting specimens and sending them to Dunedin.

Summary

1. *Latia neritoides* Gray is a pulmonate freshwater limpet from New Zealand, and is highly adapted to life in streams.

2. It is brilliantly self-luminous due to the extracellular secretion of luciferin and luciferase, and is therefore unique among freshwater animals.

3. The luminous epithelium occurs over much of the animal and is similar in appearance to that found in many marine invertebrates. It is especially similar to that of another gasteropod, Phyllirrhoë. In both species similar mucous and granular cells are found lying in the subepidermal connective tissue and projecting into the epidermis.

4. No definite function can yet be assigned to the light.

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CHLORIDE EXCRETION IN THE HEAD REGION OF FUNDULUS HETEROCLITUS

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The ability of marine teleosts to actively excrete salts from the head region was first demonstrated by Smith (1930). Although cautious in his statement, he assumed the function to be localized in the gill structure. The perfusion experiments of Keys (1931), Bateman and Keys (1932) and Schlieper (1933) proved that the gill did indeed have that function.

The identity of the cell responsible for the chloride transfer was first suggested by Keys and Willmer (1932). They gave no proof of its function, but made deductions to that end. Bevelander (1935 and 1936), after a comparative survey of many fish gills, was inclined to the view that the general respiratory epithelium, rather than any special cells, was responsible.

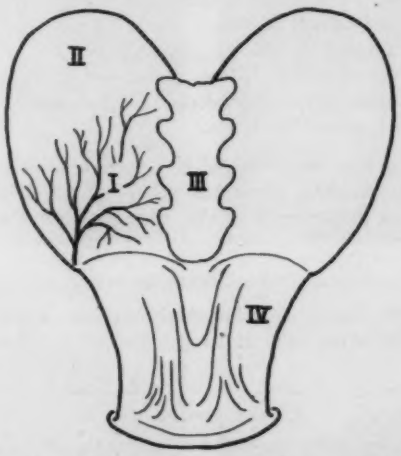


FIGURE 1. A diagram of the lower jaw with the gills removed and the opercula spread out. The normal, gross vascular supply of the operculum is shown on one side (I). The rest of the operculum (II) is relatively poorly supplied. Other areas investigated are the region of gill attachment (III) and the lateral floor of the mouth (IV).

The first significant experimental evidence was furnished by Liu (1942) who found that a certain cell type in the fresh water paradise fish, *Macropodus opercularis,* hypertrophied when the fish was exposed to gradually increased salinities. Later, Copeland (1948) gave experimental evidence that in the euryhaline *Fundulus heteroclitus* the cell homologous to those described by Keys and Willmer (1932)

and Liu (1942) is the cell responsible for chloride excretion in the gill. It has also been suggested (Pettengill and Copeland, 1948) that the same cell can reverse its polarity and be responsible for the chloride absorption observed in fresh water conditions (Krogh, 1937).

Since the chloride excreting cell of *Fundulus heteroclitus* has been investigated and identified by a number of techniques, it was decided to apply a number of them to the rest of the epithelium of the head cavities, both oral and pharyngeal, to see if the special cell is confined to the gill.

Materials and Methods

The fish used for this study were adult *Fundulus heteroclitus* which had been fully adapted to sea water.

They were killed by decapitation. The lower jaw and opercula were separated from the rest of the head and pinned down. Samples of epithelium from the numbered areas of Figure 1 were stripped from the underlying connective tissue and fixed.

Osmiophilia preparations were made following the directions of Ludford (1926).

The mitochondria were demonstrated by the method outlined by Copeland (1948) with the substitution of the following for his picric acid differentiation.

1. Differentiate with 1% methyl green, 5 sec.
2. Wash in distilled water, 10 sec.
3. Rinse in 95% alcohol, 5 sec.
4. Place in absolute alcohol I, 10 sec.
5. Finish dehydration in absolute alcohol II, 3–4 min.
6. Clear in toluene, mount in clarite.

Some of the tissue was fixed in cold 1 : 1 acetone-absolute alcohol and 7 micron sections of this material were incubated at 37° C. in 0.4% Ca glycerophosphate and handled according to Gomori's (1939) directions for the demonstration of alkaline phosphatase.

Results

Chloride cells are found distributed throughout the epithelium of the head cavity, the distribution of the cells showing a distinct correlation with the vascularity of the tissue.

Explanation of Figures

Figure 2. Preparation by the Ludford technique to show osmiophilia.

Figure 3. Alkaline phosphatase preparation by the Gomori technique.

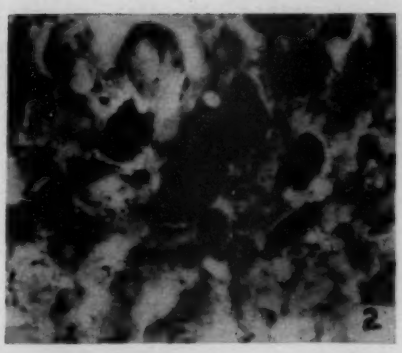
Figure 4. Mitochondrial preparation by the Regaud-Altmann acid fuchsin technique.

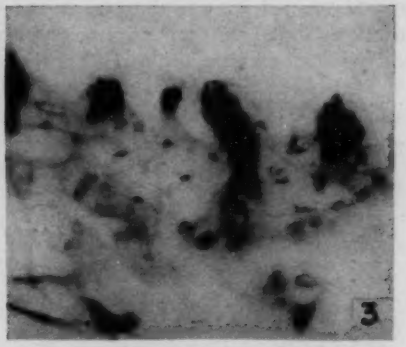
Figure 5. Epithelium from a relatively avascular region (area II of Fig. 1). No chloride cells visible in this particular view, though they are found sparsely scattered in the region. The granular cells are eosinophil granulocytes. Mitochondrial preparation.

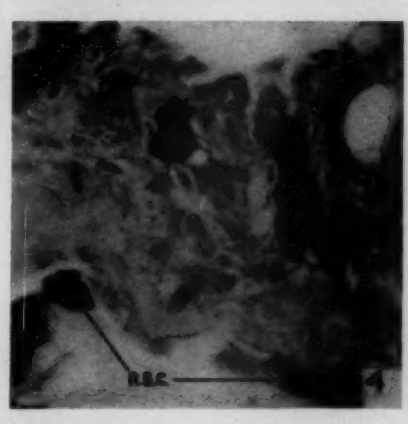
Figure 6. Epithelium from a well vascularized region (area I). Note the number of chloride cells and the proximity of the circulation. Mitochondrial preparation.

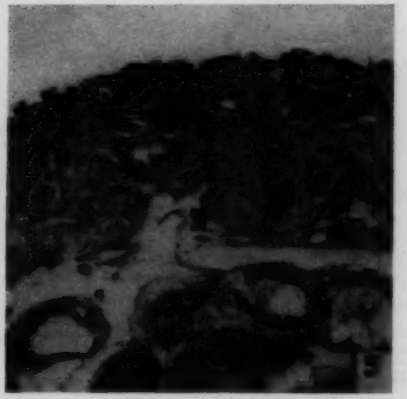
Figure 7. Epithelium from the same area as Figure 5, but from an animal that had an abnormal degree of vascularization in that area. An accompanying density of chloride cells is found. Mitochondrial preparation.

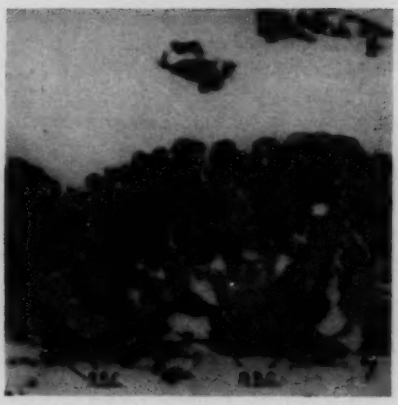
Magnification: Figures 2, 3, 4 approximately 800 × (oil immersion) and 5, 6, 7 approximately 400 × (high dry).











FIGURES 2–7

The morphology of the cells is like that of the chloride cells of the gills. The osmiophilic (Fig. 2) and alkaline phosphatase (Fig. 3) pictures correspond to those shown of the gill chloride cells by Copeland (1950) and Pettingill and Copeland (1948). The mitochondria pattern (Fig. 4) is much like that in the gills (Copeland, 1948). One dissimilarity can be noted: the cells of the gills usually contain a greater density of mitochondria.

Chloride cells are found in all parts of the cavity. In areas II, III, and IV of Figure 1 and in the roof of the mouth they are sparsely distributed, histological preparations showing many sections devoid of cells (Fig. 5). In area I they are almost as numerous as in the gill epithelium (Fig. 6).

The correlation between number of chloride cells and degree of vascularity of the epithelium is very marked. In an area of good vascularity the cells are always present in abundance (area I). The reverse is seen in regions of poor vascularity (areas II, III, IV and the roof of the mouth). That a relation exists between the cell population and vascularity of the tissue is indicated by the following observation. In one case the grossly visible blood supply extended to an unusual degree into a normally "avascular" region (area II). Histological examination revealed a large number of cells where normally few are found (Fig. 7).

Discussion

The chloride excreting mechanism in the head region of teleosts has a much wider basis than originally assumed. Chloride excreting cells similar to those in the gill filaments are found in the rest of the oral, pharyngeal, and opercular epithelium. The number in the oral cavity and in the region of the gill bar attachment (pharnygeal) is probably not great enough to play a significant part in osmoregulation. However, the relatively huge number in the opercular lining is significant.

It is very interesting that there is a correlation between the number of cells and the degree of vascularity seen in the operculum. Since the cells presumably control the salt level of the body through the mediation of the circulatory system, it is significant that such a correlation is found.

Keys and Willmer (1932) calculate that in a 250 gram eel there are 3 to 6 million chloride cells present in the gill, enough to account for the observed salt excretion. Assuming the two teleosts to be homologous in this basic arrangement, the discovery of additional cells increases the adequacy of the tissue as a mechanism for extra-renal osmoregulation.

Summary

1. The chloride excreting cell is not limited to the gill epithelium, being found in other regions of the head, especially the inner surface of the operculum.

2. With one exception, the cells appear identical in morphology and in response to Regand-Altmann, Ludford, and alkaline phosphatase techniques. The exception is that the mitochondria are usually more densely packed in the branchial cells.

3. In the operculum, the population density of the chloride cells is in positive ratio to the vascularity of the tissue. Such a topographical positioning of the cell is significant to its function of removing chlorides from the circulatory system.

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HETEROPLOIDY IN TRITURUS TOROSUS. II. THE INCIDENCE OF CHROMOSOMAL VARIATIONS IN SHIPPED LARVAE [1]

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Studies on the incidence of heteroploidy and other cytological abnormalities in salamander larvae have been given considerable impetus in recent years by the work of Fankhauser and his students. Such studies have been concerned with the experimental production of chromosomal aberrations, as well as with their spontaneous occurrence in natural populations of amphibian larvae. A general review of the results obtained before 1945 is presented by Fankhauser (1945).

The eggs of the California newt, *Triturus torosus,* are laid in "clutches" which are available in large numbers near Stanford University. Since no systematic effort had been made to determine the spontaneous incidence of heteroploidy in this form, it was decided in 1947 to undertake such a survey. Accordingly, a number of egg masses were sent by Railway Express from Palo Alto to Chapel Hill, in a thermos jug containing ice and water; this is the usual method for shipping amphibian eggs. Cytological examination of the larvae developing from these eggs revealed a high frequency of chromosomal mosaicism and very radical mitotic anomalies.

A comprehensive study was begun early in 1948 in order to eliminate the possibility that such aberrations might be present in material not subjected to shipment. Tail-tips from larvae fixed in California had only a very low incidence of abnormality, suggesting that factors involved in shipment might be responsible for the observed effects. During the period when this control material was being fixed and studied in California, another group of *T. torosus* eggs was shipped east to Chapel Hill. Egg clutches collected at the same time and in the same locale as these "1948 shipped eggs" were raised under known conditions, so that a specific control for this second group of shipped larvae was available. The data from a study of the control animals have been presented in detail by Costello and Henley (1949).

The present paper will describe the results of a study of the original (1947) group of shipped larvae, as well as the second (1948) group.

Preliminary reports concerning small random samples of these shipped larvae have been noted in abstracts (Costello and Henley, 1947a, 1947b, 1948; Costello, 1948).

We are indebted to Dr. Victor C. Twitty and Dr. H. E. Lehman for their kindness in sending us the original batch of shipped eggs in 1947.

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METHODS

1. 1947 material. Unfortunately, no data are available concerning the details of collection and shipment of these eggs. It is probable that they were in quite advanced stages of development at the time of shipment, since most of the embryos were neurulae and tail-buds when received in Chapel Hill approximately one week later. At the time of receipt, all ice in the thermos jug had melted, but the water was still cool.

Upon their arrival in Chapel Hill, the egg clutches were placed in large fingerbowls of spring water and kept in a cold room at approximately 13° C., until the time of the first tail-clipping. The eggs were removed from the cold room and allowed to return to laboratory temperature from 1 to 24 hours prior to the amputation of the tail. After this initial clipping, they were raised at laboratory room temperature, two per dish, in small fingerbowls of spring water.

At least two photographs were made of each larva, to serve as records of their appearance and gross characteristics. MS–222 or chloretone (both made up 1 : 2000 in distilled water) was used to immobilize the larvae for photographing and for the subsequent tail-clipping. The general technique utilized for amputating and staining the tail-tips has been described earlier (Costello and Henley, 1949). In many cases, three successive tips were obtained from each individual and prepared for cytological study; they were clipped at 10 to 14 day intervals. All the animals in this group were fed cladocera and Tubifex. Four hundred fifty-nine "first" tips, 338 "second" tips and 302 "third" tips were studied from these individuals.

2. 1948 material. The 1948 larvae were derived from eggs collected at Los Trancos, California, as late cleavage stages and blastulae. Fifty clutches were shipped from Palo Alto to Chapel Hill the day following collection, in "heavily iced" thermos jugs of lake water (15 ice cubes + 500 cc. lake water). Fifty other clutches collected at the same time and in the same locale were maintained at 12° to 15° C., and were kept as specific controls for the shipped eggs.

When the eggs arrived in Chapel Hill one week after shipment, they were placed in large fingerbowls of spring water. Some of the embryos were raised at laboratory room temperature, and some in the cold room, at about 6° C. After photography and the initial tail-clipping, they were assigned serial numbers and kept individually in small fingerbowls of spring water. The regenerated tail-tip and, in some cases, a "third" tip (second regenerate) were clipped and fixed at approximately 12 to 14 day intervals. Cladocera and Enchytrea were used as food for these larvae. Three hundred seventy-nine "first" tips," 269 "second" tips, and 54 "third" tips from the 1948 shipped animals were studied cytologically.

All tail-tips from both the 1947 and 1948 groups of larvae were examined, using the procedure outlined earlier (Costello and Henley, 1949). Each tip was first studied at a magnification of 210 ×; nuclear size outlines were then drawn with the aid of the camera lucida, using 10 × oculars and an oil immersion objective. Wherever possible, the number of nucleoli in each nucleus drawn was determined in the 1948 tips; the possible value of this criterion had not been foreseen at the time when the 1947 tips were studied. Chromosome counts were established using 15 × compensating eyepieces, an oil immersion objective and the camera lucida (total magnification 2100 ×).

The tips were classified utilizing the criteria discussed by Costello and Henley (1949): 1) Nuclear size and nucleolar number; 2) the presence of abnormal mitotic configurations; and 3) the presence of heteroploid metaphases checked, whenever possible, by chromosome counts. In many cases, more than one indication of abnormality was observed; under such circumstances classification was based on the most radical and clearly demonstrable of the atypical features.

Results

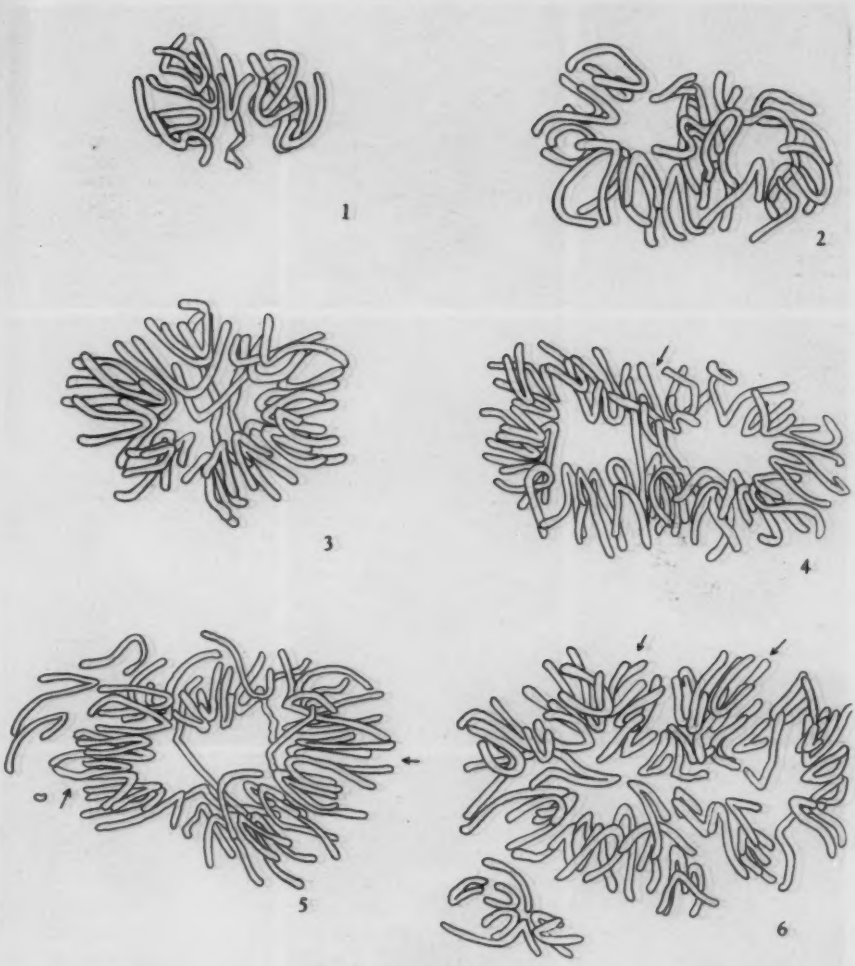
A summary of the data concerning shipped and control larvae is contained in Table I.

Table I

The incidence of cytological abnormality in shipped Triturus torosus larvae

Clipping	Total no. tips	Normal diploids		Variable nuclei		Abnormal mitoses		Mosaics	
1947 "First" tips	459	215	46.8%	213	46.4%	21	4.6%	10	2.2%
1948 "First" tips	379	166	43.8%	146	38.5%	54	14.2%	13	3.4%
Total	838	381	45.5%	359	42.8%	75	8.9%	23	2.7%
1948 Control "First" tips	582	423	72.6%	105	18.0%	53	9.1%	1	0.17%
1947 "Second" tips	338	142	42.0%	81	23.9%	39	11.5%	76	22.5%
1948 "Second" tips	269	98	36.4%	107	39.8%	55	20.4%	9	3.3%
Total	607	240	39.5%	188	30.9%	94	15.5%	85	14.0%
1948 Control "Second" tips	429	284	66.2%	88	20.5%	55	12.8%	2	0.5%
1947 "Third" tips	302	138	45.7%	126	41.7%	27	8.9%	11	3.6%
1948 "Third" tips	54	15	27.8%	33	61.1%	5	9.3%	1	1.8%
Total	356	153	43.0%	159	44.6%	32	9.0%	12	3.4%

It is apparent that the incidence of all types of abnormality is roughly comparable among the first and third tips. Furthermore, the frequency of atypical features in first and third tips of both groups of shipped larvae and in the control larvae is not nearly so pronounced as in the second tips. It has previously been pointed out that second tips are far superior to both first and third tips for cytological study (Costello and Henley, 1949), and that among control animals the occurrence of chromosomal aberrations is much more frequent in second tips. The somewhat greater incidence of abnormal mitoses among control first tips (9.1%) than in the first tips of shipped material (8.9%) is surprising. However, this apparent discrepancy can perhaps be explained by the fact that the 1948 control tips were clipped at a slightly later time than the shipped tips. They are characterized in general by a somewhat larger number of mitoses, increasing the chances that abnormalities would be noted. Among the second tips, the occurrence of cytological abnormality



EXPLANATION OF FIGURES

FIGURES 1–6. Camera lucida drawings of representative metaphase figures in tail-tips of *T. torosus* larvae subjected to shipment. The arrows indicate regions where additional chromosomes were present but too obscure to be traced along their entire length. Figure 1: Haploid; 11 chromosomes counted. Figure 2: Diploid; 22 chromosomes counted. Figure 3: Triploid; 30 chromosomes counted. Figure 4: Tetraploid; 42 chromosomes counted. Figure 5: Pentaploid; 46 chromosomes counted. Figure 6: Octaploid; 65 chromosomes counted. Drawn at a magnification of 2100×, reduced by engraver to 1250×.

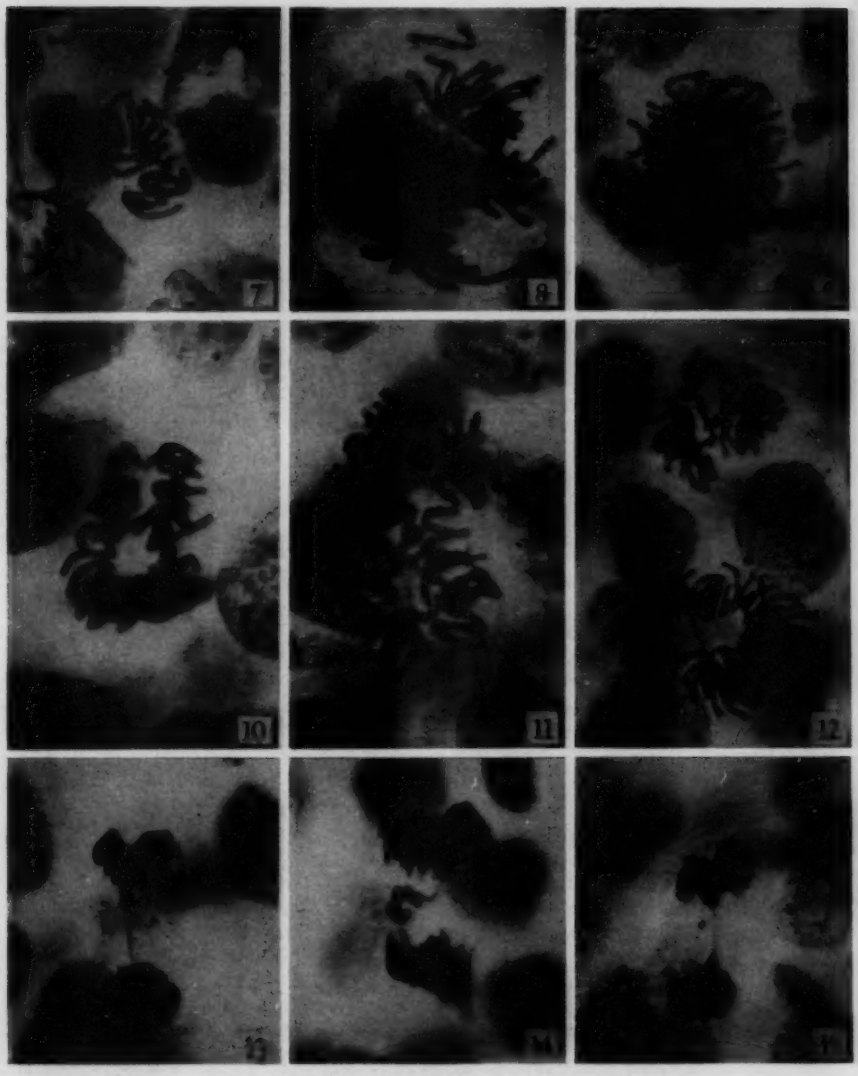


PLATE I

Photomicrographs of mitotic figures from tail-tips of shipped *T. torosus* larvae. Magnifications: Figures 7, 8, 14: 1100×; Figures 9, 12: 960×; Figures 10, 11, 13, 15: 1160×. Reduced 20% off.

FIGURE 7. Haploid metaphase (same as Fig. 1), with 11 chromosomes counted.

FIGURE 8. Pentaploid metaphase, with 46 chromosomes counted. Most of the chromosomes in the figure are split.

in the 1947 and 1948 shipped material is higher than in the control material; the number of mosaics is notably higher.

The most significant data are probably those concerned with the second tips, as indicated in Table I. Among the 338 second tips clipped from the shipped larvae in 1947, 142 (42.0%) were normal diploids, 81 (23.9%) had nuclei of variable size, 39 (11.5%) had abnormal mitoses and 76 (22.5%) were chromosomal mosaics. This is a remarkably high frequency of mosaicism, although it is lower than that reported by us in preliminary notes for much smaller random samples of these same tips (Costello and Henley, 1947a, 1947b, 1948). Of the 269 second tips studied from the 1948 shipped material, 98 (36.4%) were normal diploids, 107 (39.8%) had nuclei of variable size, 55 (20.4%) had abnormal mitoses, and 9 (3.3%) were chromosomal mosaics. The percentage occurrence of aberrations when the 1947 and 1948 groups are taken as a whole is obviously considerably greater than that recorded for second tips from control larvae fixed in 1948. Especially striking is the 14.0% incidence of chromosomal mosaicism, as compared with 0.5% for the control animals.

Mosaicism. A wide variety of types of mosaics was found. Among the 1947 first tips from shipped larvae, there were four haploid/diploids, one diploid/hyperdiploid, four diploid/triploids, and one diploid/tetraploid. Eight of these 10 mosaics were confirmed by exact chromosome counts. In all, 55 of the 76 cases of mosaicism observed among the 1947 shipped second tips were checked by direct chromosome counts. The 76 mosaics included the following: one haploid/diploid; four hypodiploid/diploids; two hypodiploid/diploid/hyperdiploids; one hypodiploid/diploid/tetraploid; 20 diploid/hyperdiploids; 13 diploid/triploids; 19 diploid/tetraploids; three diploid/pentaploids; four diploid/hexaploids; one diploid/octaploid; one diploid/triploid/tetraploid; four diploid/tetraploid/pentaploids; one diploid/tetraploid/hexaploid; one diploid/triploid/tetraploid/pentaploid; one diploid/hyperdiploid/tetraploid. Tail-tips of many of these complex mosaics showed some abnormal mitoses.

Nine chromosomal mosaics were found in the group of second tips from the 1948 shipped larvae, and 5 of these 9 were checked by chromosome counts; among them were one haploid/diploid; one diploid/hyperdiploid; 5 diploid/triploids; one diploid/tetraploid; and one diploid/pentaploid.

There were 11 cases of heteroploid figures in third tips of the 1947 material; two were checked by direct chromosome counts. The remainder were established by estimates of the number of chromosomes present in figures too crowded for exact

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FIGURE 9. Possible hexaploid metaphase. The figure was too crowded for an accurate chromosome count to be practicable.

FIGURE 10. Hypodiploid metaphase. Twenty chromosomes were counted, and the figure was sufficiently well-spread that this count is probably reliable.

FIGURE 11. Triploid metaphase, with 32 chromosomes counted.

FIGURE 12. Diploid (upper) and pentaploid (lower) (same as Fig. 5) metaphases in adjacent epidermal cells. There were 46 chromosomes counted in the pentaploid figure.

FIGURE 13. "Sticky" anaphase, with deleted fragment and bridge.

FIGURE 14. Anaphase with four lost chromosomes or chromosome fragments. At least two of these deleted elements appear to be complete or nearly complete chromosomes.

FIGURE 15. "Sticky" anaphase with a deleted chromosome fragment attached by a very fine strand of chromatin to the upper group of chromosomes.

counting. Of these 11 mosaics, one was hypodiploid/diploid; four were diploid/triploid; four were diploid/pentaploid; and two were diploid/hexaploid. The single mosaic found in the third tips from the 1948 shipped larvae was hypodiploid/diploid.

All aneuploid mosaic constitutents listed above were established by chromosome counts, since such counts are the only reliable method for ascertaining chromosome number in metaphases where only one or two chromosomes have been added to or lost from the diploid chromosome complement.

The bizarre combinations of chromosome numbers were among the striking features of the complex mosaics. In almost every case, the heteroploid metaphases occurred as isolated figures in single cells, rather than in patches of heteroploid tissue. Similarly, the sporadic occurrence of very large interphase nuclei among nuclei of normal size is evidence that, in general, the mosaic cells are not present as sizable areas of tissue. Larvae possessing these features can be characterized as predominantly diploid, but with some heteroploid cells making them mosaics.

Nuclear abnormalities. Many of the interphase nuclei showed evidence of earlier mitotic abnormality by the presence of blebs of extruded chromatin, "amoeboid" lobes, or slender connecting bridges between two adjacent nuclei. Quite frequently the periphery of the nucleus stained intensely with haematoxylin, while the central region was more nearly homogenous. Comparable configurations have been reported by us as occurring in control larvae collected from very cold water (see Fig. 17 in the paper by Costello and Henley, 1949), and in experimentally cold-treated larvae (Henley, 1950).

Mitotic abnormalities. A remarkable number of multipolar spindles was observed in tail-tips from the larvae subjected to shipment; examples are shown in

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PLATE II

Photomicrographs of epithelial nuclei and mitoses from tail-tips of shipped *T. torosus* larvae. Magnifications: Figure 16: 1100×; Figure 17: 560×; Figure 23: 960×; all other figures: 1160×. Reduced 20% off.

FIGURE 16. Nuclear débris resulting from the breakdown of epithelial nuclei. The dark globules are intensely basophilic. Small areas of such débris ("necrotic areas") are occasionally found in tail-tips from control larvae, but are not usually so extensive nor so frequent in occurrence as in shipped and experimentally cold-treated animals.

FIGURE 17. Cell boundaries in the tail-tip epithelium. This preparation was made utilizing a silver impregnation method, and the nuclei are not stained. The characteristic shape of the cells is clearly evident, as are quite pronounced variations in cell size.

FIGURE 18. Tripolar configuration of chromosomes. Superficially, this figure appears to be in anaphase, but the morphology of the chromosomes appears to be more typical of metaphase. A total of 18 chromosomes was counted. The outline of the cell boundary is quite clearly defined.

FIGURE 19. Tripolar telophase. A fine connection is present between the two upper groups of chromosomes.

FIGURE 20. Tetrapolar figure, displaced around a highly vacuolated cell. The necrotic nucleus of this vacuolated cell is visible in the center of the clear area.

FIGURE 21. Tripolar anaphase, with several persistent bridges between the two lower groups of chromosomes. The dark granules at the bottom of the photomicrograph are nuclear débris.

FIGURE 22. Tripolar anaphase, similar to that shown in Figure 18, but representing a slightly later stage. Nineteen chromosomes were counted.

FIGURE 23. Tetrapolar anaphase, in which a total of 75 chromosomes was counted. One ring chromosome is visible at the right.

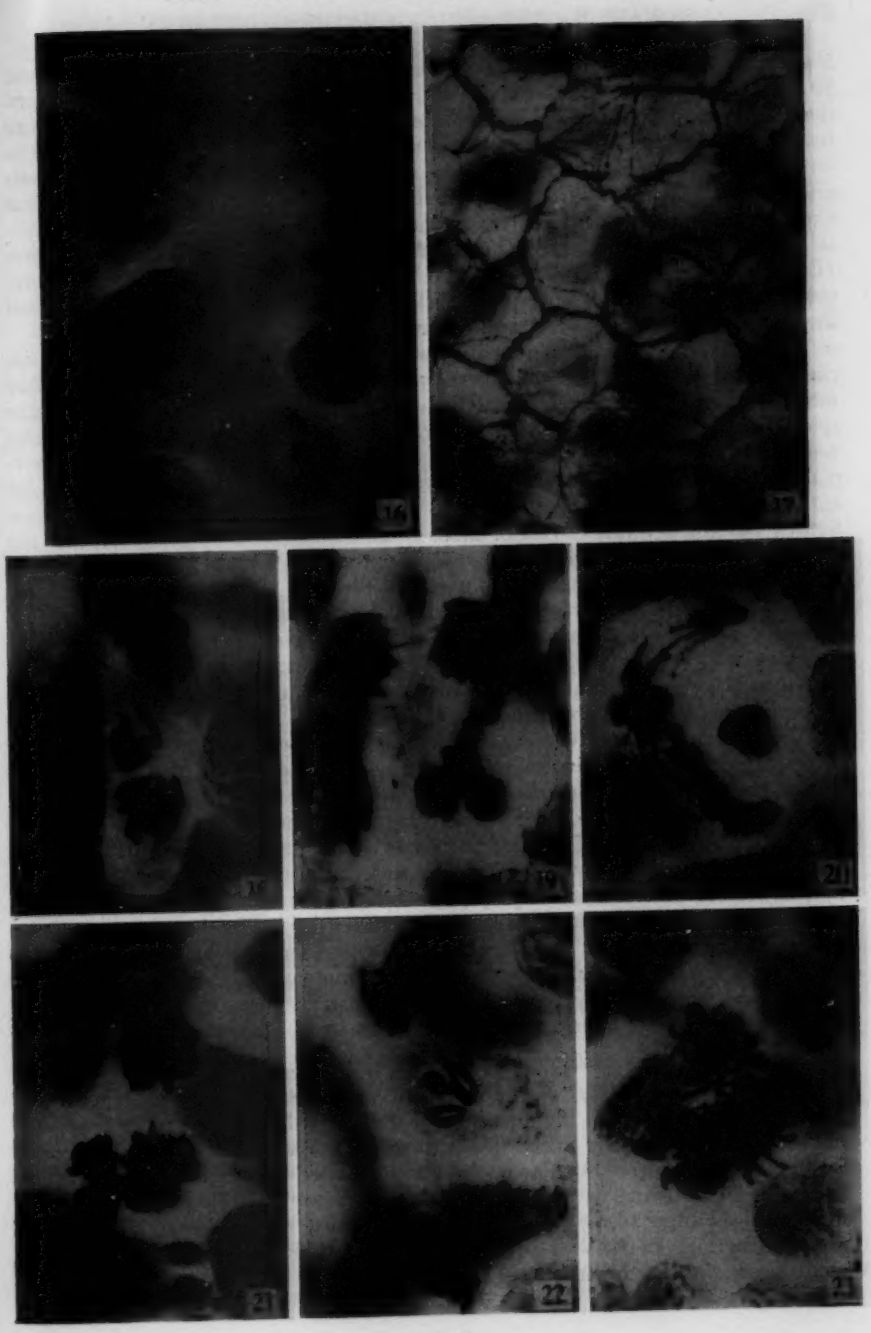

PLATE II

Figures 18–21 and Figure 23. Seven clear tripolar anaphases and telophases, and 10 tetrapolar spindles were found on first, second and third tail-tips of the 1947 and the 1948 shipped material. No such configurations have been found in tail-tips from control larvae of this species.

Another commonly observed type of aberration was the presence of relatively uncondensed masses of chromatin. Similar configurations have been reported in experimentally cold-treated *T. torosus* larvae (Henley, 1950), and are also occasionally found in control larvae, especially those collected from very cold water (Costello and Henley, 1949). These chromatin masses resemble abortive prophases. They were sometimes noted near metaphases and appeared to represent chromatin material normally present as chromosomes in a diploid figure.

A number of types of abnormality were observed in metaphases. These included "ragged" figures, in which the chromosomes were slender and thread-like rather than compact and cylindrical; the arrangement of the chromosomes on the spindle in these cases was often irregular. The chromosomes in some metaphases were intensely basophilic and arranged in dense tangled masses very unlike the normal type. Almost invariably these "wreath" configurations were too obscure for chromosome counts to be practicable. A third abnormality observed in metaphase figures was the reversal of one or more chromosomes on the spindle, so that the kinetochore region was directed outward from the center of the ring-shaped figure, rather than toward the center as is normally the case. This condition may foreshadow another frequently observed abnormality, in which the chromosomes had become completely lost from the metaphase spindle and were apparently loose in the cytoplasm. Such "lost" chromosomes or chromosome fragments have been observed in a few of the control preparations (Costello and Henley, 1949), but are not nearly so common there as in these shipped larvae.

Many types of anomalies were observed in anaphase and telophase mitoses, including the multipolar spindles referred to above. Sometimes the two poles of bipolar figures were quite markedly asymmetric, so that one had discernibly more chromatin material than the other. There were also bent spindles, in which the center of orientation of one of the two poles was almost at right angles to that of the other. "Sticky" anaphases and telophases were frequently encountered (Figs. 13, 15), as well as chromosomes and chromosome fragments which appeared to have lost their spindle connections, so that they were left behind in the migration of the daughter elements toward the poles. An extreme example of this type is shown in Figure 14. The loss or disorientation of chromosomes at the poles of anaphase or telophase figures was quite common. In a few instances, anaphases were observed in which large unorganized masses of chromatin material were present on the spindle.

Structural abnormalities of the larvae. The process of shipment appears in some way to have affected the formation of the lateral line organs in these larvae; many cases were found in which displacement of the lateral line condensations of the tail-tip had occurred, similar to those reported for experimentally cold-treated *T. torosus* larvae (Henley, 1950). Often supernumerary condensations were present toward the edges of the tail-fins; these appeared to be morphologically identical with the normal lateral line organs. The significance of these developmental anomalies is thus far obscure.

There was no observable correlation between the general morphology of the larvae and the presence or absence of cytological abnormality. A careful check of the photographs of the larvae indicated no recognizable basis for identifying larvae with either abnormal mitoses or the described types of mosaic cells. This confirms observations reported by us for control *T. torosus* larvae, as well as for experimentally cold-treated animals.

Discussion

I. Possible factors involved in the production of abnormalities

The foregoing results indicate clearly that a higher percentage of various nuclear and mitotic abnormalities is found in shipped than in control material. The major question for discussion is concerned with the reasons for such an effect. Among the possible factors contributing to the production of the abnormalities observed in the shipped material, the following may be considered:

a) *Cold.* The eggs were shipped with ice in the thermos jugs, and the temperature of the water presumably remained quite low for an extended period of time. Experimental cold-treatment has been demonstrated to produce similar anomalies in larvae of *Triturus torosus* (Henley, 1950), as well as in other species of salamanders (Böök, 1943, 1945; Fischberg, 1947; *et al.*).

b) *Partial anaerobiosis.* Utilization of most of the available oxygen within the closed jugs could have occurred during the one-week period of transit. This deficiency of oxygen might be especially pronounced near the centers of the jelly masses. There is some evidence to indicate that anoxia is of considerable importance in producing cytological abnormality in tail-tips of *Amblystoma punctatum* larvae (unpublished data).

c) *Accumulation of carbon dioxide.* The gaseous products of metabolism of the embryos do not escape from the closed jug system, from the time of shipment until the time of receipt. Such accumulation of carbon dioxide may have deleterious effects on the embryos, either directly or indirectly, through lowering of the pH of the medium.

d) *Mechanical agitation.* Shipment by Railway Express probably involves almost continuous shaking of the jug contents.

e) *Toxic water.* The ice used was made by freezing ordinary tap water; even though the liquid medium was non-chlorinated lake water (also used for raising the control animals), the water from the melting ice may have introduced toxic factors. In general, it is the experience of the Stanford group of embryologists that larvae which are raised in heavily chlorinated or other unsuitable water are characterized by laterally-curled tail-tips, with deviated tail-fins. No such curled tails were noted in our material, so perhaps this factor may be eliminated from further consideration.

f) *Irradiation.* This remote possiblity is mentioned only because of the similarity between the observed aberrations and the types of irradiation-produced anomalies described by Alberti and Politzer (1924a, 1924b). We have no evidence that any such radiations were present, and it would be a remarkable coincidence if they were operative, even in different dosages, during two separate salamander breeding seasons.

g) Anaesthesia and other factors inherent in the process of photographing the larvae. Theoretically, the anomalies found might be attributed to the effects of the anaesthetic required to immobilize the larvae for photography, or to the heat given off by the Photoflood lamps used as a light source. Such effects, if present, would be expected to bear a definite relation to the time of subsequent clipping. However, they can probably be ruled out, on the basis of cytological studies of tail-tips from *Amblystoma punctatum* larvae which were not subjected to anaesthesia or photography (unpublished data).

II. The correlation between cytological abnormality present on first, second and third tail-tips

On the assumption that the factors responsible for mitotic anomalies were present during the period of egg shipment, as indicated above, the data were examined to ascertain the degree of correlation between the occurrence of abnormalities in first, second and third tail-tips. If the factors producing abnormality were operative during the period of late cleavage, gastrulation and neurulation, we should expect to find some regularity in the subsequent events taking place in the epidermal cells of the growing larvae. There are several possibilities: 1) All three tail-tips of an affected larva might have a similar high percentage of abnormal figures. 2) The three tail-tips could exhibit a progressive increase in the number of abnormal figures, due to an increase in the number of cell progeny of the affected cells. 3) There might be a progressive decrease in the number of atypical mitoses in the three tips. This would obtain if the abnormal cells ceased to divide further and, becoming necrotic, were eliminated from the tip.

The data indicate that very little correlation exists. Only three second tail-tips were mosaic among the 23 larvae whose first tips were characterized by that condition. From this same group of 23, not even one third tail-tip was found to have heteroploid metaphases. Of the 85 larvae with mosaic second tips, three belonged to the group of 23 with mosaic first tips. A single animal of the group of 85 showed mosaicism in its third tail-tip. Only one individual of the 12 which had mosaic third tips was demonstrated to have had heteroploid figures present before the time of the third clipping.

In addition to this lack of correlation, there was no progressive increase or decrease in abnormality. Instead, the incidence of atypical figures was found to be much higher in second tips (85 mosaics) than in "firsts" (23) or "thirds" (12). How can this be explained?

The second tips are by far the most favorable material, for several reasons. They are relatively free of yolk, whereas the first tips may have some yolk-obscured cells, especially if clipped a little too early. Furthermore, the incidence of mitosis is very much higher in the second tips than in either "firsts" or "thirds." This may be due to the onset of a period of rapid growth and cell multiplication concurrent with the beginning of feeding; such a period presumably tapers off after about 10 days. Another possibility is that there is more rapid cell division following the stimulation of the initial tail-clipping, which persists for as much as 12 to 14 days. However, if this be true, it seems strange that a similar stimulation of mitosis does not follow the second amputation. Finally, one must consider the fact that the second tips have noticeably fewer pigment cells than the "firsts" (but not

fewer than the "thirds"), so that a smaller number of figures would be obscured. This finding, in itself, obviously does not account for the differences between the "seconds" on the one hand, and the "firsts" and "thirds" on the other.

The frequent occurrence in this material of necrotic areas, with nuclear débris resulting from partial or complete disintegration of epithelial nuclei, is an established fact. A typical example of this phenomenon is figured (Fig. 16). The staining characteristics of such areas are described briefly by Costello and Henley (1949). We have no way of knowing whether or not nuclear necrosis is initiated in abnormal cells. It appears that arrested nuclear disintegration could be an effective cause of hypodiploid nuclei.

It must be concluded that, at the present time, we cannot adequately account for the high percentage of deviations from normal mitotic conditions. Neither can we account for the higher incidence of abnormalities found in the 1947 shipped material, as compared with the larvae subjected to shipment in 1948. We do know, however, that the 1948 embryos were kept under more carefully controlled conditions before shipment. If the radical aberrations described herein are the result of such minor environmental changes as obtain in the ordinary procedures for handling and shipping embryological materials, it is of vital importance to emphasize this fact. Many amphibian embryologists working in this country today are experimenting with cytologically sensitive and perhaps abnormal material.

Summary

1. Tail-tips from *Triturus torosus* larvae subjected to shipment by Railway Express have been studied cytologically.

2. A total of 838 "first" tips was examined. Of these, 381 (45.5%) were normal diploids, 359 (42.8%) had nuclei of variable size and/or variations in nucleolar number, 75 (8.9%) had abnormal mitoses, and 23 (2.7%) were chromosomal mosaics.

3. Of the 607 regenerated "second" tips studied, 240 (39.5%) were normal diploids, 188 (30.9%) had nuclei of variable size and/or variations in nucleolar number, 94 (15.5%) had abnormal mitoses, and 85 (14.0%) were chromosomal mosaics.

4. Three hundred fifty-six second regenerates ("third" tips) were examined. Among these, 153 (43.0%) were normal diploids, 159 (44.6%) had nuclei of variable size and/or variations in nucleolar number, 32 (9.0%) had abnormal mitoses, and 12 (3.4%) were chromosomal mosaics.

5. The following types of mosaics were found: haploid/diploid; hypodiploid/diploid; hypodiploid/diploid/hyperdiploid; hypodiploid/diploid/tetraploid; diploid/hyperdiploid; diploid/triploid; diploid/tetraploid; diploid/pentaploid; diploid/hexaploid; diploid/octaploid; diploid/triploid/tetraploid; diploid/tetraploid/pentaploid; diploid/tetraploid/hexaploid; diploid/triploid/tetraploid/pentaploid; diploid/hyperdiploid/tetraploid. These mosaics were predominantly diploid, with, for the most part, isolated heteroploid cells in scattered areas.

6. Seventeen instances of multipolar anaphases and telophases were found in the tail-tips from these shipped larvae. Seven of these were tripolar and 10 were tetrapolar.

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SPERM ENTRANCE IN ECHINODERMS, OBSERVED WITH THE PHASE CONTRAST MICROSCOPE[1]

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In most animal species it is an accepted fact that the entire spermatozoan, including the tail, enters the egg to accomplish fertilization and, in many cases, has been demonstrated within the egg cytoplasm of stained sections (Fig. 1) (Selenka, 1878; Meves, 1915; Yatsu, 1909; Kostanecki and Wierzejsky, 1896). In echino-

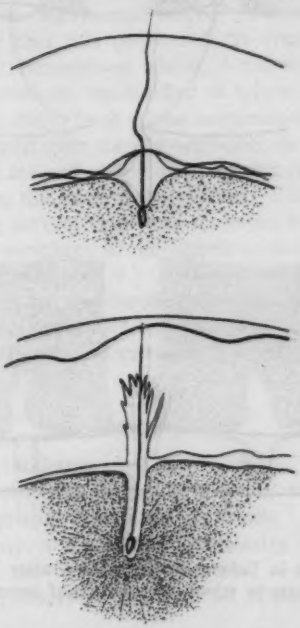

FIGURE 1. Sperm penetration into the egg of *Toxopneustes variegatus*, as drawn from living material by E. Selenka in 1878.

[1] The phase contrast microscope used in this and the following study was purchased with the aid of a grant from the American Philosophical Society in 1947. The writer wishes to express sincerest thanks to the Society, in particular to Prof. E. G. Conklin, and to the Bausch and Lomb Optical Company, for their exceedingly kind cooperation in making the instrument available ahead of their production schedule, at the expense of considerable inconvenience and extra effort on the part of their staff.

[2] The writer also wishes to thank the Director and Staff of the Misaki Marine Biological Station for the use of Station facilities.

derms, however, Wilson (1895) found the sperm tail being carried away on the outside of the rising fertilization membrane (Toxopneustes) and he was unable to detect it within the cytoplasm of either living or fixed eggs. Consequently, although he cites the work of Ries (1909) to the contrary in his book (which since 1925 has served as the definitive treatise of cytology), his lack of certainty in the matter has created a general impression that echinoderms are an exception to the rule that the entire spermatozoan enters the egg.

The later work of R. Chambers (1933) with *Echinarachnius parma* confirms Wilson's observations. Chambers found that the tail of the partially engulfed spermatozoan is gripped by the expanding fertilization membrane and either car-

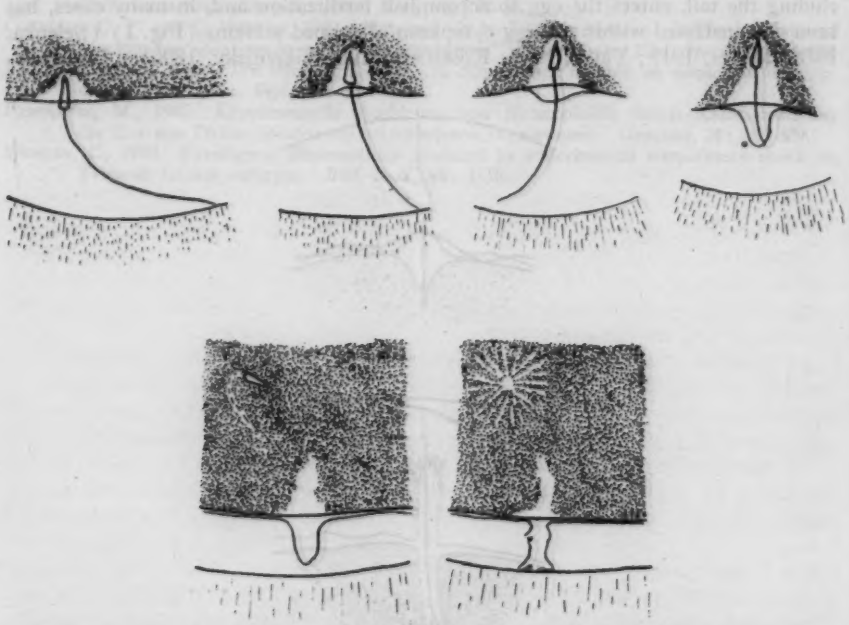

FIGURE 2. Sperm penetration in *Echinarachnius parma*, after R. Chambers, 1933. Chambers describes this as "a case in which the sperm was unusually active" (p. 133).

ried away in entirety from the egg surface as in Wilson's figure, or held until the advance of the head portion has created sufficient tension to cause the tail to break at some point within the membrane. Chambers sidesteps the final decision as to whether any part of the sperm tail enters the egg cytoplasm, by stating that the truncated portion of the tail was "lost among the processes of the exudation cone" (p. 132). However, Chambers presents one case, involving an "unusually active" spermatozoan, in which ". . . the entire length of the sperm tail came to lie within the membrane," and eventually was visible moving within the egg cytoplasm (Fig. 2).

In view of the important status held by echinoderm eggs as a result of the volume of research which has centered about them, there seemed sufficient reason for bringing improved microscopical technique to bear on the settlement of this old discrepancy in observation.

Material and Methods

Since the phase contrast microscope permits highly accurate observation of such objects as living spermatozoa, it was used in a comparative study of spermatozoan entry in the echinoderm species readily available at the Misaki Marine Biological Station. These include the sea urchins, *Strongylocentrotus pulcherrimus, Heliocidaris crassispina, Clypeaster japonica, Mespilia globulus* and *Pseudocentrotus depressus,* and the starfish, *Asterina pectinifera.*

In order to observe the entire process of insemination, the following procedure was adopted. A small drop of sea water containing the eggs to be fertilized was placed on a glass slide on the microscope stage. A cover glass was coated with saliva because it was found that this prevented the trapping of the spermatozoa against the glass surface—a phenomenon which, if not checked, simultaneously interfered with visibility and reduced the number of sperm available for fertilization.

A very small amount of a dilute fresh sperm suspension was spread on the coated cover glass, and this was placed over the egg-sea water drop. With practice it was found possible to control the amount of sea water so that there was very little movement of the eggs by the time the focus was adjusted. Using a medium low power objective, a field containing several eggs was surveyed to catch a sperm about to enter an egg exactly on the periphery of the largest optical section; the process was then followed with the high dry (43 ×) or the oil immersion (97 ×) objective. Best observation of the sperm tail and fertilization cone was achieved with the high dry objective. It was ordinarily possible to begin observations within about 20 seconds, just as the spermatozoa were penetrating the jelly and approaching the egg surfaces.

Results

In the six species of echinoderms used in this study, it was found that without exception the entire spermatozoan, including the tail, entered the egg in a continuous process, usually requiring from 2 to 4 minutes.

For the sake of continuity, athough this necessitates repetition of facts already well known to students of the fertilization process, a composite account will be presented of the phenomena observed in any or all of the species under study.

Penetration of the vitelline membrane

As the spermatozoa begin to arrive at the surface of the unfertilized egg, pushing through the jelly with a slightly rotational swimming movement, one will be seen in which the head, almost immediately after reaching the egg, assumes a position perpendicular to the surface. Although the tail may persist in its vigorous motion, there is no communication of this to the head, which seems to have established a rigid connection with the egg protoplasm, presumably having already made an opening in the metaplasmic vitelline membrane.

In spite of the fact that *Asterina* is a starfish and its eggs are in the germinal vesicle stage when insemination takes place, there was no significant difference observed in the manner of sperm entry between it and the five sea urchin species.

Wave of cortical granule breakdown

The reaction of the egg to the presence of the spermatozoan which has thus made contact with its living protoplasm begins at once, with a visible wave which travels around the egg at speeds varying with the species, but in those measured (*Mespilia, Pseudocentrotus*) roughly of the order of 10 μ sec. This is undoubtedly the "wave of cortical granule breakdown" of Moser, 1939; Motomura, 1941; Runnström, 1948.

In the relatively fluid eggs of Mespilia, the passage of this wave is especially striking; it causes a slight deformation of the surface layers of the egg, which gives the impression that some sort of tension is being progressively released, or that a local band of contraction and expansion is passing around the egg. The effects of this phenomenon are not confined to the outermost layers; eggs which have been stratified and elongated by centrifugation lose much of their stratification and regain a spherical contour as a result of the passage of this wave.

FIGURE 3. *Pseudocentrotus depressus.* The first stage of sperm entrance is characterized by vigorous writhing activity which draws the entire length of the tail within the fertilization membrane as soon as a small perivitelline space has been formed. During the second stage, the tail is relatively quiescent as it is gradually engulfed in the egg cytoplasm.

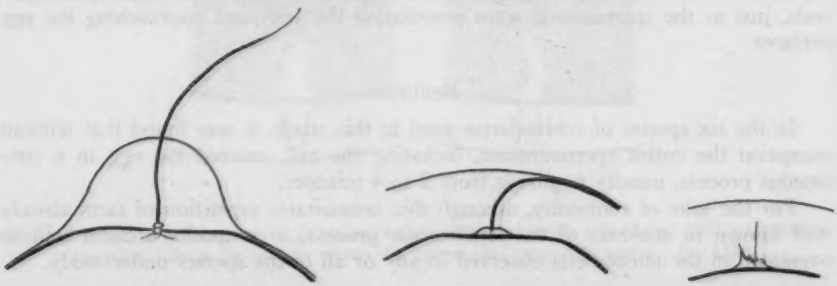

FIGURE 4. *Clypeaster japonica.* In this form the fertilization membrane is lifted rapidly, but the relatively inactive sperm tail is not invariably pulled into the perivitelline space. The originally rounded fertilization cone develops a ragged appearance in the course of degeneration.

Elevation of the fertilization membrane

In four of the species—*Mespilia, Clypeaster, Pseudocentrotus* and *Strongylocentrotus*—a rapid separation of the fertilization membrane is immediately initiated around the point of sperm entry, while the wave of cortical granule breakdown is proceeding around the remainder of the egg. Over an area represented by a little

less than one fourth of the egg circumference in optical section, the egg surface is flattened (by the back-thrust of a swelling colloid which pushes up the fertilization membrane?); in the center of this area the sperm head is being steadily drawn into the egg cortex.

At the same time, the fertilization membrane, which has been "loosened" around the egg by the first wave, is gradually being pushed away from the protoplasmic surface.

In *Heliocidaris* and *Asterina*, the fertilization membrane is slower in separating, and the perivitelline space does not attain as great a width as that of the other four species.

FIGURE 5. *Mespilia globulus.* The fertilization membrane lifts rapidly when the egg is in optimal condition, but the sperm tail may extend through the membrane while it is being drawn into the egg cytoplasm. The degenerating fertilization cone usually has several fine processes.

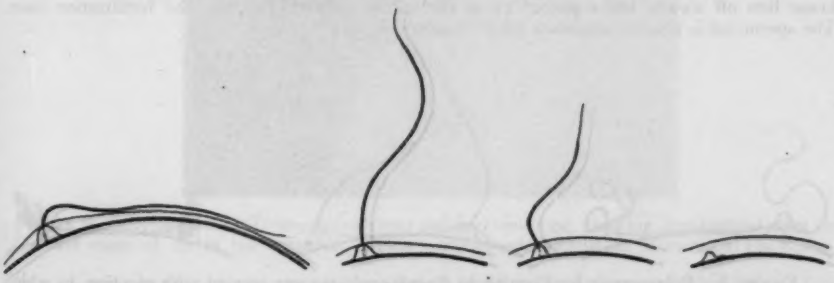

FIGURE 6. *Heliocidaris crassispina.* After the sperm head has penetrated the cortex, the tail lies motionless on the outside of the fertilization membrane for about two minutes; it then straightens out perpendicular to the egg surface and is rapidly drawn into the cytoplasm.

Fertilization cone

The fertilization cones formed in all six species are remarkably similar during what is presumably their functional period (see Figs. 3, 4, 5, 6, 7, 8, 9); *i.e.*, while the sperm tail is passing through them into the egg cortex. In each case the cone develops as a mound of hyaline substance after the sperm head has been engulfed into the cytoplasm, and degenerates more or less promptly after the tail has been engulfed.

The shapes taken by the degenerating cones differ rather widely among the various species, from the irregular knobs of *Heliocidaris* (Fig. 6) to the spikes and processes produced by *Clypeaster* (Fig. 4) and *Mespilia* (Fig. 5). *Heliocidaris* and *Asterina* (Fig. 7) are alike in that the fertilization cone develops rapidly to full

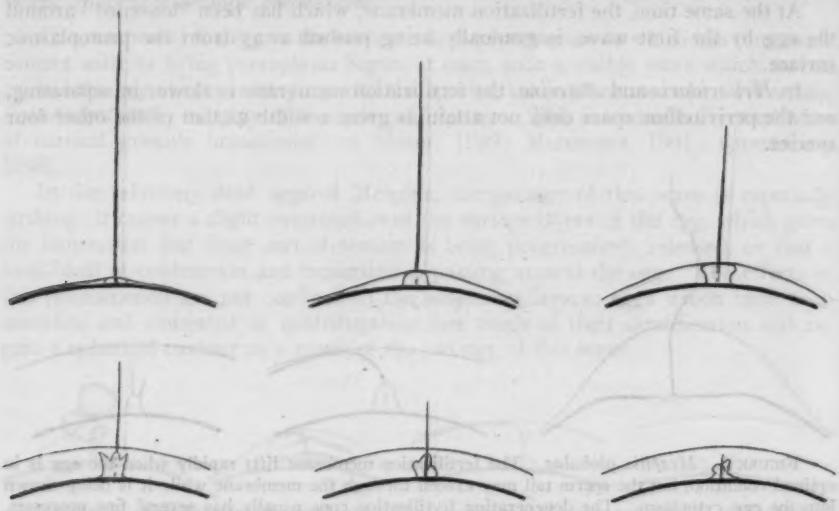

FIGURE 7. Sperm entrance in the starfish, *Asterina pectinifera*. The fertilization membrane lifts off slowly, being pushed up at the sperm entrance point by the fertilization cone. The sperm tail is nearly motionless while it enters the egg.

FIGURE 8. Polyspermic fertilization in *Pseudocentrotus* egg treated with nicotine, in which the fertilization membrane is not lifted from the egg surface and abnormally large and persistent fertilization cones are formed. No change from the normal pattern can be detected in the activity of the spermatozoan.

size and pushes away the slowly separating fertilization membrane at the point of sperm entrance; in the other species full development of the rounded stage is attained somewhat later. The fertilization cones of *Strongylocentrotus* and *Pseudocentrotus* (Fig. 3) subside, or are resorbed into the surface layers of the egg without any spectacular developments in normal cases, although elaborate processes resembling bundles of hyaline fibers were produced in *Pseudocentrotus* eggs treated with nicotine (Fig. 8), and in untreated, immature eggs. Heating to 35° C. also caused the formation of abnormally large cones in *Clypeaster* eggs.

Sperm tail entrance

The degree of activity exhibited by the tails while the spermatozoa are entering the egg cytoplasm varies widely among the species studied. As the consequence of a vigorous writhing motion, the tail of the *Pseudocentrotus* sperm is invariably pulled into the perivitelline space as soon as it is formed. In contrast to this behavior, the sperm tail activity of *Clypeaster, Mespilia* and *Strongylocentrotus* ranges from negligible to spasmodically vigorous, and the tails are often found extending through the fertilization membrane until the length of tail remaining outside the egg becomes less than the width of the perivitelline space. In no species was there

FIGURE 9. Photograph of *Strongylocentrotus pulcherrimus* egg, showing fertilization cone and last stage of sperm tail entrance. Phase contrast microscope, oil immersion (97×).

observed any phenomenon which could be interpreted as indicating that the fertilization membrane exerted any restraining effect on the sperm tail at any time. From the observations made on these six species it must be assumed that the aperture made in the outermost covering of the egg at the moment of sperm head penetration remains as such, so that the much more slender tail of the sperm is entirely free to slip through it.

In *Heliocidaris* and *Asterina*, since the perivitelline space is slow to develop and never attains as great a width as that of the other four species, the sperm tail invariably remains outside the fertilization membrane until it is finally engulfed in the fertilization cone (Figs. 6, 7) after about 3½ minutes.

The sperm tail entrance pattern of *Heliocidaris* shows an interesting variation peculiar to its species. After the head has been engulfed, the fertilization membrane

lifted off and the fertilization cone formed, the sperm tail, instead of gradually being drawn into the egg, remains lying motionless against the outside of the fertilization membrane (Fig. 6) until about 2½ minutes after fertilization; it then suddenly straightens out to a position perpendicular to the egg surface and, moving vigorously, almost the entire length of the sperm tail enters the egg within 15 to 30 seconds. This is the fastest rate observed in any of the species in which the entrance time was recorded.

The time records for the other species, in both of which the tail slowly enters the egg at an approximately uniform rate, are also shown in Table I. These data seem to indicate a faster rate of entrance with rise in temperature within the normal range. However, the data are insufficient to permit the drawing of any conclusion, in view of the amount of variation observed among eggs of the same lots.

TABLE I

Time required for complete engulfment of sperm tail

Species	No. cases	Temp. °C.	Minimum	Maximum	Average
Clypeaster	40	22°–24°	125 sec.	355 sec.	196 sec.
	45	26°–28°	120 sec.	265 sec.	183 sec.
Mespilia	39	27.5°–30°	170 sec.	660 sec.	———
Heliocidaris	18	21.4°–23°	90 sec.	360 sec.	161 sec.
	13	28°	100 sec.	160 sec.	122 sec.

It will be noticed that no average time is given for *Mespilia*. In this form, in about half of the cases observed, the sperm tail was engulfed at a steady rate within 3½ minutes. In the remaining cases, the engulfment of the tail required from 4 to 11 minutes, and, in a few instances, as in Chambers' experience, the tip of the tail "was lost among the processes of the entrance cone," so that it was impossible to fix an accurate time of final engulfment.

Regardless of this desultory behavior on the part of its tail, the sperm head showed no corresponding lag in the formation of the sperm aster and union with the egg pronucleus.

An attempt was made to cause an increase or decrease in the time required for engulfment of the sperm tail in *Heliocidaris*, *Clypeaster* and *Mespilia* by subjecting the eggs to various abnormal conditions such as chilling (8° C.), warming (35° C.), aging and exposure to polyspermy-producing agents. In no case was a significant change in the entrance time observable. In cases of polyspermy in which two or three sperm entered the egg simultaneously, the entrance time for all the spermatozoa was approximately equal and within the normal range.

During the period covered by the sperm tail entrance into the egg, there are two phenomena which attract the attention: one is the appearance of crystal-like objects in the perivitelline space of *Pseudocentrotus* and *Strongylocentrotus* eggs and the other is a marked "surging" of the entire *Clypeaster* egg mass. The first phenomenon has been intensively studied by Y. Endo, whose report on the subject will be published in the near future.

"Surging" of the cytoplasm—Clypeaster

This mass movement of the cytoplasm has no counterpart, so far as could be determined, among the other species studied, and can hardly be associated with the wave of cortical granule breakdown, since it is initiated after the head of the sperm has completely penetrated the egg cortex—*i.e.*, about 40 seconds after the time of cortical granule breakdown. Starting at this time, the whole mass of egg protoplasm is carried toward the part of the surface where the sperm is being engulfed, so that the contour bulges out in this region. After 30 to 40 seconds this movement slows to a stop, and then immediately begins in the reverse direction, continuing for approximately the same period, again slowing to a stop, reversing its direction and returning to the original equilibrium position in another 30 to 40 seconds. The egg mass has thus completed one period of something approximating simple harmonic motion; and at its conclusion the sperm tail is usually just disappearing into the egg.

This phenomenon is not observed to occur equally in all *Clypeaster* eggs, even under normal (experimental) conditions. It was especially marked in eggs inseminated at 8° C., and did not occur at all in eggs which were fertilized after being kept at 35° C. for one hour. It took place whether or not the fertilization membrane had previously separated to a normal extent, and it was not prevented, and possibly even enhanced, by compressing the eggs moderately under a cover glass.

Movement of the sperm head within the egg

In the exceedingly transparent eggs of the summer urchins *Clypeaster* and *Mespilia*, it is possible, with the phase contrast microscope, to observe the sperm head after it has penetrated the cortex and begun its progress toward the center of the egg. The appearance of the head and middle piece is the same as that outside the egg, but it has been impossible so far to detect the tail with certainty inside the egg at this stage.

Whether the cause of the apparent over-all lability of these eggs lies in a highly fluid state of the hyaline ground-substance, a relative paucity of granular inclusions, or a combination of the two conditions has not been studied. However, with the high dry phase contrast objective, the cytoplasm of the unfertilized egg is seen to consist of small currents of endoplasm flowing at random among undefined stationary regions which seem to consist of loose aggregations of various-sized granules and vesicles exhibiting different degrees of density and refringence.

In such a milieu, the sperm head is distinguished by its being larger than any one of the granular inclusions and often markedly refringent. Under the most favorable optical conditions its elongated triangular shape is clearly visible. However, its detection would be extremely difficult without the spasmodic movements which it performs. Once it has been brought into focus, its progress can be followed for two or three minutes while it moves through the cytoplasm with what appears to be a combination of jerky movements on its own part and the effect of local protoplasmic streaming on the part of the egg. In the region around the entering spermatozoan the random streaming characteristic of the unfertilized egg becomes directed to some extent, with a column of noticeably centripetal flow surrounding the path of the sperm.

Whenever simultaneous observations of the sperm head and tail were possible, it was found that movement of one was always associated with a corresponding

movement of the other. In both these species (*Mespilia* and *Clypeaster*) the sperm tail, while relatively inactive, exhibits spasmodic movements from time to time, and as a result of these, the sperm head pushed about among the granules, sometimes causing the displacement of an aggregation two or three times its own diameter. Often it was seen to turn through 90 or more degrees, moving to the side and in one case, going back almost to its entrance point.

This activity continues for a period that roughly corresponds to the time required for engulfment of the sperm tail, although it has not been possible to discover any causal relation between the two, and there is actually evidence against such a conclusion. In half the cases of sperm entrance recorded for *Mespilia*, the complete cessation of movement of the sperm head, followed immediately by the disappearance of its membrane and the appearance of sperm astral rays took place while a sometimes considerable portion of the sperm tail was still visible outside the egg. In a single example of this behavior in *Clypeaster*, the sperm aster appeared at 4'20" (about the usual time), while half the length of the tail was still extending through and well past the fertilization membrane.

These observations coincide perfectly with the one exceptional case described by Chambers (see Fig. 2). Until a phase contrast study is made of Chambers' material (*Echinarachnius parma*) it will be impossible to determine with certainty whether this situation is actually an exception, or whether it is the rule as it has been found to be in the six other echinoderm species under discussion.

Formation of sperm aster—behavior of middle piece

Whatever factors set the time for the end of the spermatozoan's existence as an independent gamete, it occurs with relative constancy about four minutes after the beginning of fertilization in both the species in which the process is clearly visible. At this time, the outline of the sperm head fades from sight, its refringence is lost, and within 20 to 30 seconds its place is occupied by a hyaline mass without well-defined boundaries, from which small hyaline rays begin to extend into the surrounding cytoplasm.

After the disappearance of the sperm head membrane, the middle piece can be seen lying in the egg cytoplasm close to the hyaline pronuclear mass. As the astral rays elongate and presumably force the pronuclear mass toward the center of the egg by pushing against the egg cortex and/or surface layers (E. L. Chambers, 1939), the middle piece travels with it, near the center of the aster. It appears as a vesicle at the upper limit of the size range of ordinary granular inclusions, containing highly refringent material which appears variously as one or two masses or as a ring of material apposed to the inner surface of the vesicle (probably depending upon the direction from which it is observed as it is pushed about by the movement of the cytoplasmic inclusions surrounding it).

It continues to be recognizable without change through the first cleavage, lying near or even against the membrane of the fusion nucleus in approximately the future equatorial plane (*i.e.*, about equidistant from the two poles of the amphiaster), and taking no detectable part in the various changes which precede cleavage. It is finally carried as an inert cell inclusion into one of the blastomeres when the cell divides.

A complete account of the fate of the *Parechinus miliaris* sperm middle piece, obtained from a study of fixed material, was presented by Meves in 1912 and 1914.

The present phase contrast observations are offered only as confirmation of Meves' painstaking and beautiful work, and as giving such final conclusiveness as comes from following the process in one living cell. Meves carried his observations through the fifth cleavage, at which time he found the still unchanged middle piece in either a macro- or a mesomere of the 32-cell-stage larvae.

Union of sperm with egg pronucleus

In the course of the study of sperm heads within *Mespilia* egg cytoplasm, one case was observed in which the spermatozoan entered in the immediate vicinity of an exceptionally excentric egg pronucleus, and worked its way toward the center of the egg for approximately a minute and a half, until it came to within about 5 μ of the pronuclear membrane. At this juncture it was suddenly carried sideways across the intervening space and held against the pronuclear membrane, its own membrane intact and its tail moving as it was gradually drawn into the egg at the usual rate. The sperm head maintained its integrity of form during this period (ca. 2 min.), at the end of which disintegration of its membrane and appearance of the sperm aster took place as in ordinary cases.

Discussion

Several rather interesting problems have been touched on in the course of this study, such as the actual fate of the sperm tail, especially the exact process by which it is taken into the egg; the nature of the stimulus which sets off the fertilization reaction of the egg; the nature of the forces determining the time at which the spermatozoan terminates its life as an independent unit and becomes an integral part of the fertilized egg; the nature of the attracting forces between the egg and sperm pronucleus; and the nature of the stimulus which directs the course of cytoplasmic streaming in the neighborhood of the entering spermatozoan. Since, however, discussion of most of these questions at this time would involve a preponderance of speculation, and since further study should shed the more reliable light of fact on at least some of them, they are suggested here mainly as inducements to further investigation.

The process by which the sperm tail enters the echinoderm egg may be more involved than a first impression would indicate. In no case does the sperm head penetrate into the cytoplasm more than 20 μ from the entrance point during the three or four minutes while the tail is being engulfed; this is difficult to reconcile with the fact that the length of the tail in these species varies between 40 and 55 μ.

The exceptional case of *Mespilia*, in which the tail was very slow in entering some eggs, contributes only complication to this problem, since, even supposing that the tail remains attached in some way to the sperm pronucleus which takes the place of its head, and is gradually drawn after the synkaryon as it moves to the center of the egg, the radius of the egg minus the radius of the synkaryon is clearly less than the length of the tail, notwithstanding which fact the entire tail eventually disappears within the egg.

Given the apparatus for satisfactory observation of spermatozoa, there are certain conclusions which can be drawn from their behavior. It is obvious that the first sperm to reach the surface of an unfertilized egg is not necessarily the one which finally enters it. It is also accepted as fact that, in echinoderm eggs, any point on the surface is capable of receiving a sperm and setting off the fertilization reaction.

It can then be concluded that some optimal condition of vitality in certain spermatozoa gives them the capacity to effect a local change in the outermost living layer of the egg which sets off a receiving reaction on the part of the egg as a whole.

It seems safe to assume further that the spermatozoan capable of setting off such a reaction has been actively able to penetrate the vitelline membrane of the unfertilized egg in order to reach the living, reactive protoplasm. Direct evidence in favor of such a conclusion is obtained from observation of the fertilization process in the egg of *Ostrea gigas,* which has a relatively resistant vitelline membrane. In order to penetrate this membrane, the round-headed spermatozoan maintains a vigorous rotating attack which continues for from six to ten minutes before connection is established with the underlying cytoplasm. In sea urchins, this connection seems to be made almost instantaneously, or within one or two seconds—at least several hundred times faster than in oysters. Since the difference in the thickness of the vitelline membranes does not seem to be of that order, it is a temptation to place this observation among the evidence in favor of the idea that the sea urchin sperm secretes a substance from the tip of the acrosome (Popa, 1927) which has a locally destructive effect on the vitelline membrane.

As a sequel to the observation of the sperm head being attracted to the egg pronucleus, *Pseudocentrotus* eggs were crushed in artificial Ca-free sea water so that the contents flowed out freely. The pronuclei remained intact and recognizable under these conditions, appearing under the phase contrast oil immersion objective as non-refringent, perfectly spherical and completely empty vesicles. When sperm were added to this suspension, they swam freely about, often adhering temporarily or permanently to one or another of the various cytoplasmic inclusions and sometimes attaching themselves to the egg pronuclei. However, they showed no "preference" for pronuclei over other bodies, and were often observed approaching pronuclei in a random fashion, pushing them about for a while, and wandering off again. While it is not suggested that these conditions imitate those within living egg cytoplasm, the observation is offered for such negative value as it may have.

What can be stated positively from this observation is that the attraction between the egg and sperm pronuclei antedates the appearance of the sperm-astral rays, and that syngamy can take place without their intervention. Moreover, it was found that this pattern of sperm entrance and syngamy, which is exceptional in sea urchins, is the invariable rule in the medusan, *Spirocodon saltatrix* (Dan, 1950). The spermatozoan enters the egg in the immediate vicinity of the egg pronucleus, and syngamy takes place without the formation of a sperm aster, so far as could be determined by the most careful phase contrast observation of these exceedingly transparent eggs.

Summary

1. Phase contrast microscope observation of sperm penetration into the eggs of five species of sea urchins—*Mespilia globulus, Clypeaster japonica, Strongylocentrotus pulcherrimus, Pseudocentrotus depressus, Heliocidaris crassispina*—and one starfish—*Asterina pectinifera*—have shown that total engulfment of the spermatozoan including all of the tail, is the rule in these species.

2. In every case the fertilization cone makes its first appearance after contact has been established between the sperm head and the living egg cytoplasm, and persists in normal cases for from 5 to 15 minutes after the beginning of sperm penetra-

tion. Subjection of the eggs to some experimental conditions—heat, cold, nicotine solution—causes abnormal production and persistence of the fertilization cones.

3. Engulfment of the sperm tail in these species is usually complete within 2–4 minutes after the beginning of penetration. No significant change in engulfment time could be induced by exposing the eggs to the abnormal physiological conditions tested—heat, cold, aging, exposure to polyspermy-producing agents.

4. In the two species with the most transparent eggs (*Mespilia* and *Clypeaster*), the movements of the sperm head within the cytoplasm can be clearly observed, from the time it passes the cortex until it becomes quiescent, which approximately coincides with the final engulfment of the tail. Progress of the spermatozoan appears to be effected by a combination of autonomous movement and streaming of the egg cytoplasm.

5. About four minutes after the beginning of fertilization, the clear outline of the sperm head is lost and the sperm aster begins to develop. From this time the middle piece of the spermatozoan can be found lying in the cytoplasm, and traced as it moves together with the sperm aster toward the center of the egg. So far as could be determined, it takes no active part in the first cleavage.

6. In one case a sperm head, which had entered an egg close to the exceptionally excentric egg pronucleus, was attracted to and held motionless against the pronuclear membrane, within about a minute and a half after entering the cytoplasm, while its tail was still largely outside the egg. This, however, produced no variation in the subsequent process of engulfment of the tail and formation of the sperm aster.

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FERTILIZATION IN THE MEDUSAN, SPIROCODON SALTATRIX

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Spirocodon saltatrix, a bell-shaped member of the *Tubularioanthomedusae* between four and eight centimeters in diameter, appears in the inner waters of some of the deeper bays near the Misaki Marine Biological Station in the beginning of January. The young adults carry fully developed eggs and sperm from the time of their first appearance, and during the succeeding three months the animals breed constantly, gradually increasing in size and shifting their habitat from the heads of the bays toward the open sea. At the middle of March most of the animals found are at the upper limit of the size range and contain large quantities of eggs and sperm. There is a brief period during which the gametes are obviously not in optimal condition, after which the medusae completely disappear from the waters near the Station. Nothing has as yet been learned concerning the whereabouts of this species during the remaining nine months of the year.

The embryology of *S. saltatrix* has not been investigated because of the failure so far to rear larvae past the gastrula stage or to collect intermediate stages. A method of inducing spawning was developed by Uchida (1927) and elaborated by Kume (1948). Since these animals spawn soon after nightfall, placing them in the dark in the laboratory causes a copious release of mature gametes within approximately an hour, regardless of the day-night relationship. It has been found that a previous exposure of ten to fifteen minutes to the direct rays of an ordinary lamp improves the reactivity to the stimulus of darkness.

Examination of eggs taken from the ovaries during the morning and early afternoon reveals only immature eggs, whereas spawned eggs are always fully mature. It is thus apparent that the light-darkness change is the stimulus for the beginning of the maturation divisions, and eggs removed from the ovaries of females which have been so stimulated go through the maturation divisions on the microscope stage.

All attempts to fertilize such eggs by adding freshly shed sperm to them have, however, so far failed, as have methods such as mixing separately shed egg and sperm suspensions, or adding sperm suspension to "dry" eggs shed from ovaries cut from animals just before spawning time. It is thus obvious that the eggs are fertilizable for only a very short time after their expulsion from the ovaries, although the specific reason for this has not yet been determined.

In order to obtain eggs in the earliest stages of the fertilization process, the following procedure was adopted: several medusae, after removal from darkness, were placed in sea water in separate containers and observed closely. (Since the males invariably spawn first, it was possible to differentiate between the sexes without undue handling of the animals.) Females just before spawning time were placed either in a container with a shedding male or in sea water containing freshly shed sperm, and watched for the beginning of spawning. Since the spermatozoa also lose their fertilizing capacity after a very brief exposure to sea water, con-

siderable care must be exercised to ensure the presence of freshly shed sperm in the sea water into which the eggs are to be shed. As soon as eggs were observed in the container, a sample was removed to a slide and observed with the phase contrast microscope.

Mature, unfertilized eggs (Fig. 1) are slightly oval with a depression at the animal pole, where the polar bodies have just been extruded, and at the base of which the pronucleus lies in contact with the egg surface. One or both of the polar bodies may sometimes be found still adhering to the polar region, although these are usually lost in the course of shedding and the handling of the eggs prior to observation. There are no enveloping layers or membranes, so far as can be determined, either before or after fertilization.

FIGURE 1. Mature unfertilized egg of *Spirocodon saltatrix*. The egg pronucleus lies at the base of a slight depression left after the extrusion of the second polar body.

The fertilizing spermatozoan invariably enters the egg in the immediate vicinity of the egg pronucleus. Further evidence for the existence of some sort of specific attraction on the part of the egg pronucleus for the free-swimming spermatozoa is found in the fact that excess sperm are found only around the animal pole of mature eggs, even in the case of eggs which have been rendered unfertilizable by contact with sea water before the addition of sperm suspension.

Immediately after the penetration of the sperm head into the egg cortex, there develops, around the sperm tail, a tubular structure (Fig. 2a) which gradually increases in both length and diameter for about five minutes (Fig. 2b–e), while the sperm tail, which maintains slight but continuous movement, is steadily being drawn into the egg. The walls of this "fertilization tube" are entirely transparent, and the sperm tail is perfectly visible moving freely within the tube. In several cases a second spermatozoan was found with its head well within the tube.

After attaining approximately the size shown in Figure 2e, the tube begins a course of degeneration, during which it continues to expand in size without, apparently, any increment in material. In fact, there seems to be a chemical dissolution of the structure, as though sea water were an unfavorable medium for its existence. Breaks appear in the walls, and the lashing of the shortening sperm tail causes further disintegration. Especially if there are other spermatozoa around the animal pole of the egg, their movements usually tear the now balloon-shaped structure from its place of attachment, and it gradually becomes more and more attenuated, although fragments of its can sometimes be found after 20–30 minutes.

Complete engulfment of the tail usually requires about 15 minutes. While this is occurring, the egg becomes spherical, and the indentation at the animal pole disappears. The egg pronucleus slowly leaves its original position directly under the surface, moving a short distance toward the center of the egg and showing a marked

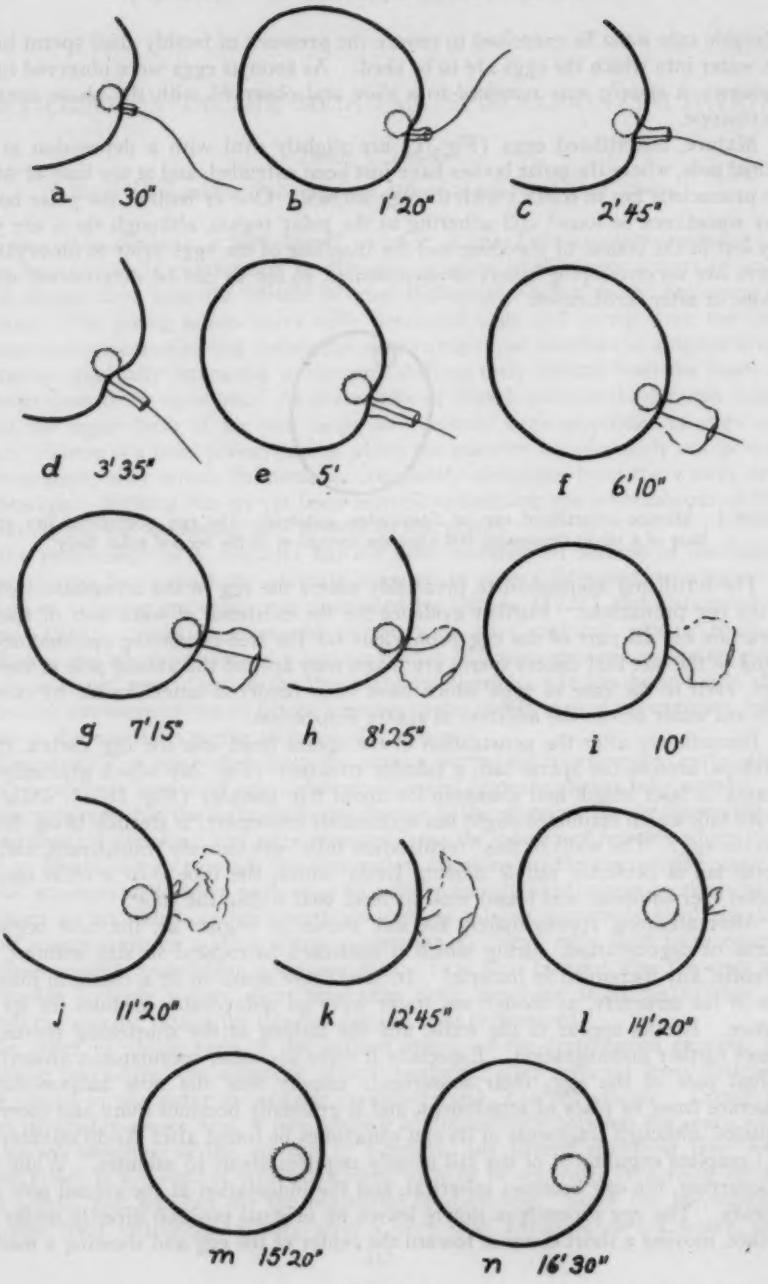
a 30"
b 1'20"
c 2'45"
d 3'35"
e 5'
f 6'10"
g 7'15"
h 8'25"
i 10'
j 11'20"
k 12'45"
l 14'20"
m 15'20"
n 16'30"

FIGURE 2

increase in size. Although the most careful observation of these remarkably transparent eggs failed to reveal any trace of the sperm head after it entered the egg, it seems safe to conjecture that the union of the sperm pronucleus has occurred, without astral activity, between 10 and 14 minutes after the beginning of fertilization. This would account for the increase in nuclear size noted above.

Soon after the stage shown in Figure 2n, the outline of the nucleus disappears entirely, to be followed shortly by the appearance of the cleavage diaster, in the same excentric position previously occupied by the fusion nucleus. For a description of the cleavage pattern, reference is made to Dan and Dan, 1947.

Summary

Eggs of the tubularioanthomedusan, *Spirocodon saltatrix*, which breeds in the neighborhood of the Misaki Station from the beginning of January to the latter part of March, are spawned daily, shortly after nightfall. The change from light to darkness causes the initiation of the maturation divisions, at the completion of which the eggs are shed. Spawning can be induced by darkening in the laboratory.

In the mature eggs, the pronucleus lies in contact with the surface, at the base of a slight depression at the animal pole. These eggs are fertilizable only when spawned into sea water containing freshly shed spermatozoa.

Examination of such eggs with the phase contrast microscope shows that the fertilizing sperm always enters in the immediate vicinity of the egg pronucleus. At the sperm entrance point a tubular structure of transparent substance begins to develop around the sperm tail as soon as the head of the sperm has penetrated the egg. This continues to increase in length and diameter for about five minutes, after which a process of degeneration sets in. At the end of about fifteen minutes the tube has become balloon-shaped and is more or less rapidly disintegrating, as the tip of the sperm tail is finally drawn into the egg.

The egg pronucleus moves a short distance from the periphery toward the center of the egg. A marked increase in size between 10 and 14 minutes after the beginning of fertilization probably indicates the union of the sperm and egg pronuclei, although no astral activity is detectable.

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Figure 2. Camera lucida drawings of sperm entrance in *S. saltatrix*. (Time recorded from beginning of observation.) (a) Immediately after engulfment of sperm head. First appearance of "fertilization tube." (b–e) Increase in length and diameter of fertilization tube, sperm tail gradually being drawn into egg. (f) Beginning of degeneration of fertilization tube, disappearance of polar depression. (f–h) Egg pronucleus beginning to move inward. (i–m) Increase in size probably indicates fusion of egg and sperm pronuclei. Sperm tail completely drawn into egg, remnant of fertilization tube lost. (n) Typically spherical condition of fertilized egg achieved.

OXYGEN CONSUMPTION, BRAIN METABOLISM AND RESPIRATORY MOVEMENTS OF GOLDFISH DURING TEMPERATURE ACCLIMATIZATION, WITH SPECIAL REFERENCE TO LOWERED TEMPERATURES[1]

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On passing from one temperature to another within the range of viable temperatures, a poikilothermic animal undergoes rapid alterations in its physiological processes. This rapid change is followed by a slower series of changes in the direction of the original state, and a final condition is reached which, in most cases, is intermediate between the original state and that reached shortly after the temperature change. The second, slower series of changes is known as temperature acclimatization.

Among fish, acclimatization may be accompanied by changes in the temperatures compatible with life (Loeb and Wasteneys, 1912; Hathaway, 1927; Fry, Brett and Clawson, 1942; Doudoroff, 1942, 1945; Brett, 1944, 1946), changes in the metabolic rate (Wells, 1935a, 1935b), changes in the rate of respiratory movements (Sumner and Doudoroff, 1938), and changes in the optimum temperatures for sustained swimming (Fry and Hart, 1948).

Though many and extensive studies of temperature acclimatization have been made (Belehradek, 1935), the fundamental processes involved have not been made clear.

On the basis of field observations, Agersborg (1930) suggested that the death of carp (*Cyprinus carpio*) and of gizzard shad (*Dorsoma copedianum*) after passing from water at 28° C. to 30° C. into a lake at about 0° C. is due to respiratory failure. Adrian and Buytendijk (1931) found rhythmic potential changes in the isolated goldfish brain stem which are similar in timing to the respiratory movements. They conclude that the respiratory movements in this fish are manifestations of an inherent rhythm in the central nervous system. In mammals at least, such rhythmic potential changes in brain tissue are dependent upon the metabolic rate of the tissue (Himwich *et al.*, 1939; Hoagland, 1949). In view of this, we may surmise that alteration in the respiratory rhythm results from changes in brain metabolism. If an increase in brain metabolism were found to occur during low temperature acclimatization, it might account for the increase in the rate of respiratory movements and might also show a correlation with the increase in activity and in oxygen consumption which accompany such acclimatization.

Accordingly, the correlation of brain metabolism with total oxygen consumption and with respiratory movements has been examined, first in goldfish previously

[1] A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Duke University, Durham, N. C. The author wishes to express his appreciation to the University Council on Research of Tulane University for a grant for continuation of this study, some data from which are included in this article.

acclimatized at several different temperatures and then in fish undergoing acclimatization to a lowered temperature.

Materials and Methods

Animals. Goldfish, *Carassius auratus*, obtained from Beldt's Aquarium, St. Louis, Mo., were used throughout the study. The fish ranged in size from 1.5 to 3 inches, exclusive of the caudal fin. On arrival, fish were placed directly in chambers held at the experimental temperatures or into holding tanks until space was available in the experimental chambers. A week or longer was allowed for acclimatization if the previous holding temperature was within 3° of the experimental temperature. Otherwise, the acclimatization period was two weeks or longer.

The fish were fed daily with Beldt's "Best Goldfish Food." Oxygen determinations on the water in which fish were kept indicated that oxygen concentrations were not limiting under the conditions of the experiments (Toryu, 1927; Fry and Hart, 1948).

Temperature control. Temperatures were maintained within 0.1° using thermoregulators. Temperatures below room temperature were maintained by constantly cooling and intermittently heating with thermostatically controlled heaters.

Oxygen consumption of fish. In observations at 37.5° C., the change in oxygen concentration of water passing at a constant rate through a small darkened chamber containing the fish was used in calculations of oxygen consumption. The method is similar to that used by Keys (1930). In observations at other temperatures, the change in oxygen concentration of water in a closed chamber in which the fish were held overnight was used for determinations of oxygen consumption. The method is similar to that used by Ege and Krogh (1915). Corrections were made in both cases using chambers without fish but otherwise treated in a similar manner. Oxygen concentrations were determined by the standard Winkler titration (American Public Health Association, 1946).

Oxygen consumption of brain tissue. Fish were taken from the water and the brains exposed immediately by removal of the roof of the skull. The brain was severed posterior to the vagal lobes, lifted out and blotted briefly on filter paper to remove blood and meninges. Brains from at least two (and usually three) fish were pooled, weighed together to 0.2 mgm., ground in a dry mortar and taken up in phosphate buffer of pH 7.5 prepared by mixing 0.160 *M* KH_2PO_4 and 0.121 *M* $NaHPO_4$.[2] The breis thus prepared were transferred to Warburg flasks with sufficient buffer to bring the volume to 3.00 ml. For CO_2 absorption, 0.20 ml. 10% KOH solution was placed in the central well of each flask along with a piece of folded filter paper. About ten minutes elapsed between the removal of the first fish from water and mounting of the flask and manometer on the Warburg apparatus.

After allowing ten minutes for thermal equilibration, readings were made at ten minute intervals at temperatures other than 0° C.; at 0° C. readings were made at 30-minute intervals. Corrected pressure changes for five intervals were plotted against time on graph paper and the best fitting line drawn "by eye" to obtain a slope for use in calculation of the rate of oxygen utilization.

Rates of opercular movements. Rates of opercular movements were determined by timing fifty movements of each of ten to twenty fish at 12°, 20°, 27° or 37.5° C.

[2] The effects of added glucose on brain brei metabolism will be reported elsewhere.

between 7:30 and 9:30 A.M. At 4° C., ten movements were timed on each of four fish on five consecutive days.

Results

Certain aspects of the metabolism of goldfish completely acclimatized to several temperatures were first examined. Specifically, oxygen consumption of the fish and of brain and muscle tissues were measured and the rates of opercular movements were studied. The oxygen consumption of brain tissue from fish acclimatized at two temperatures was studied over the temperature range from 0° to 37.5° C. These data served as the basis of a subsequent study of changes during the course of acclimatization.

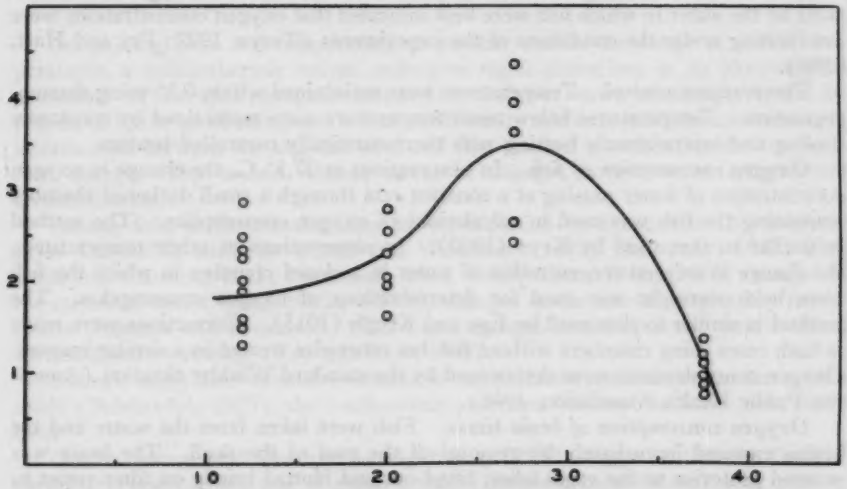

Figure 1. Oxygen consumption of goldfish at acclimatization temperatures. Ordinate: oxygen consumption in mgm./kg./min.; abscissa: temperature (C.).

Oxygen consumption of fish at acclimatization temperatures. Data on the oxygen consumption of whole fish at the acclimatization temperatures (Fig. 1) exhibit a maximum at temperatures around 27° C., though there is considerable variation at each temperature studied.

Brain tissue respiration. Brain tissue respiration was studied as possibly correlated with respiratory movements and with total oxygen consumption and overall activity of the fish.

Breis from the brains of fish acclimatized at 12°, 20°, 27°, and 37.5° C. were studied at 18.9° C., a temperature near the center of the range of temperatures tolerated by goldfish. Use of this temperature also avoids the possibility of heat inactivation of brain enzymes. Throughout the range studied, an inverse relation is found between acclimatization temperature and the metabolic activity of brain tissue breis measured at 18.9° C. (Fig. 2). Between 12° and 27° C. the

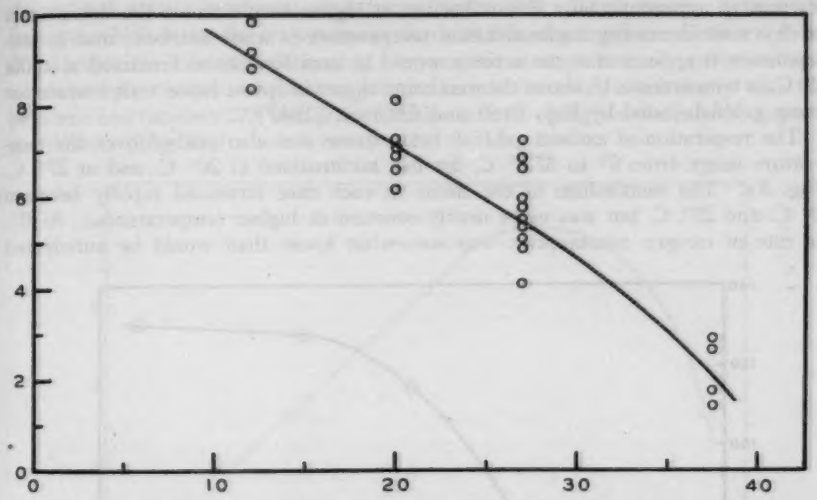

FIGURE 2. Oxygen consumption at 18.9° C. of brain tissue from fish acclimatized at various temperatures. Ordinate: oxygen consumption in mgm./kg. brain tissue/min.; abscissa: acclimatization temperature.

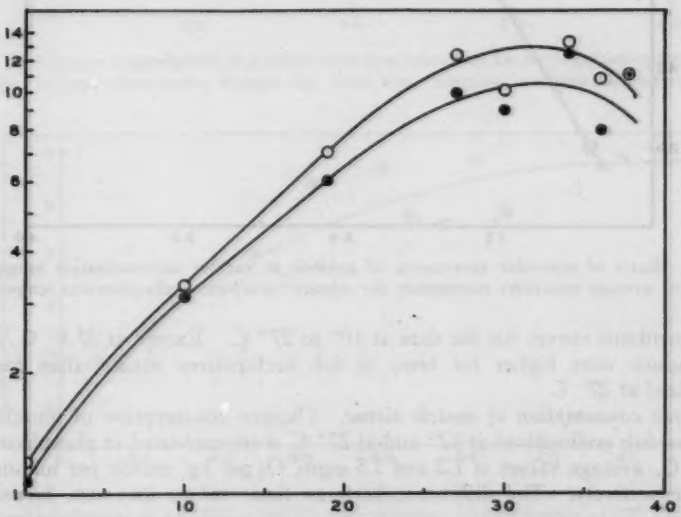

FIGURE 3. Oxygen consumption at various temperatures of brain tissue from fish acclimatized at 20° C. and at 27° C. Acclimatization temperatures: 20° (○); 27° (●). Ordinate: oxygen consumption in mgm./kg. brain/min.; abscissa: temperature.

relationship appears to be a linear one but at higher temperatures the decrease in activity with increasing acclimatization temperature is more marked; and by extrapolation it appears that the activity would be zero for fish acclimatized at about 42° C., a temperature 1° above the maximum upper incipient lethal temperature for young goldfish found by Fry, Brett and Clawson (1942).

The respiration of excised goldfish brain tissue was also studied over the temperature range from 0° to 37.8° C. for fish acclimatized at 20° C. and at 27° C. (Fig. 3). The metabolism of the tissue in each case increased rapidly between 10° C. and 27° C. but was more nearly constant at higher temperatures. At 0°, the rate of oxygen consumption was somewhat lower than would be anticipated

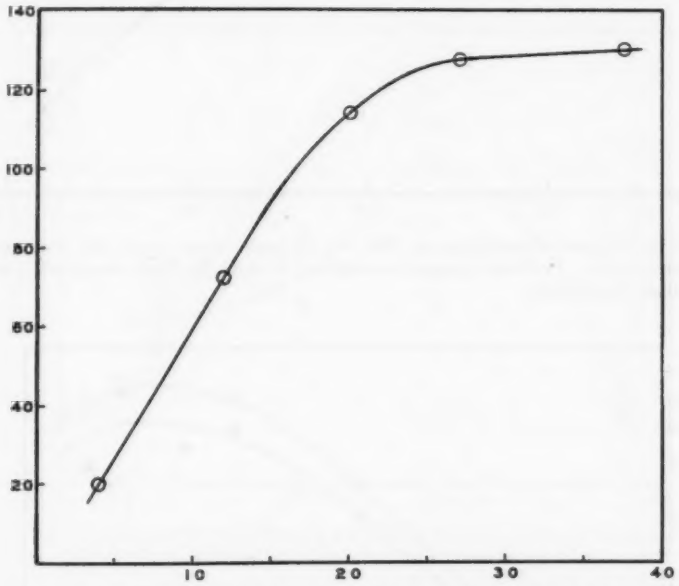

FIGURE 4. Rates of opercular movements of goldfish at various acclimatization temperatures. Ordinate: average opercular movements per minute; abscissa: acclimatization temperature.

from logarithmic curves for the data at 10° to 27° C. Except at 37.8° C., oxygen consumptions were higher for breis of fish acclimatized at 20° than for those acclimatized at 27° C.

Oxygen consumption of muscle tissue. Oxygen consumption of muscle tissue breis from fish acclimatized at 12° and at 27° C. were measured in phosphate buffer at 18.9° C., average values of 1.2 and 1.5 mgm. O_2 per kg. muscle per minute being found, respectively. The difference between these values may not, however, be significant.

Opercular movements at acclimatization temperatures. Rates of opercular movements were studied at acclimatization temperatures 4°, 12°, 20°, 27°, and 37.5° C. as a function which is normal and necessary to the fish and which may be

determined with a minimum disturbance to the fish. Furthermore, it may be a function which limits the temperatures at which fish may survive. Between 4° and 20° C. there is a linear relationship between the average rate of opercular movements and the temperature; above 20° C. the rate increases more slowly with temperature and between 27° and 37.5° C. there is little change in the rate of opercular movements (Fig. 4).

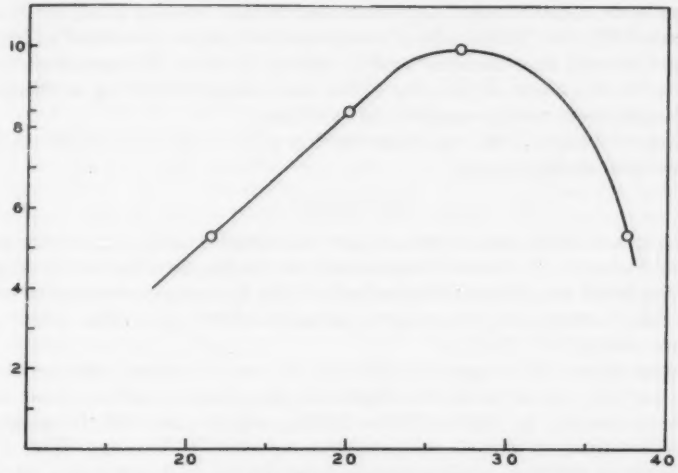

FIGURE 5. Oxygen consumption of goldfish brain brei calculated for acclimatization temperatures. Ordinate: oxygen consumption in mgm./kg. brain/min.; abscissa: acclimatization temperature.

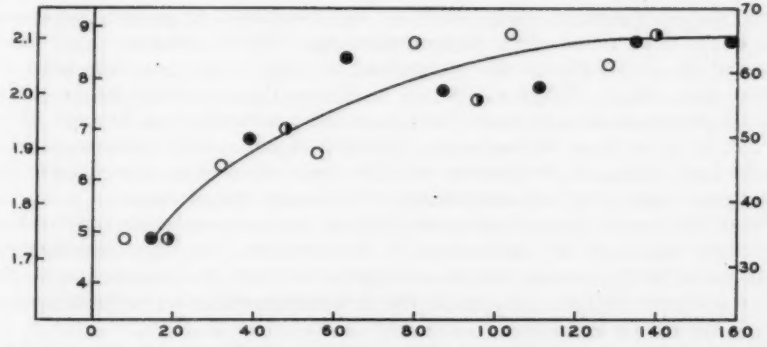

FIGURE 6. Rates of opercular movements, oxygen consumption of fish and of brain tissue during acclimatization of goldfish at 12° C. after previous acclimatization at 27° C. Ordinates (left to right): oxygen consumption of fish in mgm./kg./min. (●); oxygen consumption of brain brei in mgm./kg./min. (◑); and average rate of opercular movements per minute (○); abscissa: hours after transfer from 27° C. to 12° C.

Change of oxygen consumption of fish and of brain tissue and of opercular movement rates during acclimatization. Curves for the rates of opercular movements (Fig. 4), brain respiration at acclimatization temperatures (Fig. 5) and of oxygen consumption of fish at the acclimatization temperatures (Fig. 1) show marked similarities. In order to test these relationships further, the rates at which these change during acclimatization of fish at 12° C., after previously having been acclimatized at 27° C., have been observed. Observations were made at 24 and 48-hour intervals until values were obtained which approximated in each respect those for fish fully acclimatized at 12° C. The results of these observations are presented in Figure 6. The changes in each case occurred rapidly during the first 48 hours after transfer from 27° to 12° C., about 50 per cent of the total change occurring in this period; and the changes were nearly complete in four days.

The rate of change of the opercular rhythm after transfer from 20° to 27° C. was studied with similar results.

Discussion

The changes in brain metabolism, oxygen consumption and in opercular rhythm during acclimatization to lowered temperature are in the direction which might account for the better adaptation of the animals to the low temperatures after acclimatization. The increase in each respect partially offsets the initial effect of the temperature change.

The observations on oxygen consumption of the fish agree with previous observations on the Pacific killifish (*Fundulus parvipinnis*) and on mud suckers (*Gillichthys mirabilis*) by Wells (1935a; 1935b) and, in part, with those on young goldfish acclimatized at various temperatures reported by Fry and Hart (1948). Similar relations between acclimatization temperature and opercular rates have also been reported for the mud sucker by Wells (1935b) and by Sumner and Doudoroff (1938).

Only two reports of studies of the metabolism of fish brain tissue have appeared, that of Fuhrman *et al.* (1944) on the metabolism at graded temperature levels of the brain tissue of the largemouthed bass (*Huro salmoides*) and that of Field and Peiss (1949) on the metabolism of brain tissue from the polar cod (*Boreogadus saida*). These results are similar to those obtained in the present study on the metabolism of brain breis from fish acclimatized at 20° and 27° C. The cod, living at lower temperatures, exhibited higher rates of brain metabolism than the bass; just as in the present study the brain metabolism at a common temperature was highest for fish acclimatized at the lowest temperatures.

When fish respiration and opercular rhythms are compared with rates of brain metabolism calculated for acclimatization temperatures, the depression in brain metabolism at 37.5° C. is found to be intermediate between that for total metabolism and the opercular rhythm. In view of this it is notable that after acclimatization at 37.5° C. the fish are less active in other respects than those at 27° C.

Previous attempts to relate the metabolic changes of the whole organism during acclimatization to the metabolic activity of a single tissue or organ have failed. The present study indicates, however, that there is an inverse relationship between the level of activity of the excised brain tissue and the acclimatization temperature of the goldfish, and that the rate at which brain metabolism changes during temperature acclimatization is of the same order as that for opercular movements and

fish oxygen consumption. Further, the oxygen consumption of brain tissue over the range from 0° to 27° C. is very similar to that for goldfish as observed by Ege and Krogh (1915) and by Gardner, King and Powers (1922). In neither case, however, can the direct effect of temperature on muscle account for the differences in whole animal respiration, for there are no significant differences in the metabolism of muscle tissue from fish acclimatized at 27° C. and at 12° C.; and Morales (1943) has shown that respiration of frog muscle tissue changes but little with changes in temperature over this range. It would appear that a major factor in determining the level of oxygen consumption of the fish is the metabolic activity of the brain, acting through its influence on the other tissues of the body.

At the three lower temperatures, 12°, 20°, and 27° C., the relation between the average brain respiration and the average rate of opercular movements is a direct one; the ratios between brain brei respiration calculated for the acclimatization temperatures and the observed rates of opercular movements being constant over this temperature range. There are similarities between the curves for the total oxygen consumption of the fish (Fry, 1947) and of brain metabolism calculated for the acclimatization temperatures. There are also comparable variations in the curves for fish and brain tissue metabolism at temperatures below 10° C., and, as previously mentioned, at temperatures near 37.5° C. Such similarities are especially significant in view of the close correlation between the metabolic activity of brain tissue and its rhythmic activity (Himwich *et al.*, 1939; Hoagland, 1949).

The long-term response of the goldfish to a lowering of the environmental temperature is an increase in metabolism and of general activity above the level of unacclimatized fish at the same temperature, an increase having obvious adaptive advantages. The increase in brain metabolism during low temperature acclimatization may be one of the homeostatic mechanisms making possible the maintenance of normal functioning to a higher degree than would otherwise be possible.

Summary

1. Oxygen consumption of goldfish at acclimatization temperatures increases between 12° C. and 27° C. but is again lower at 37.8° C.

2. When measured at a single temperature, there is an inverse relation between oxygen consumption of brain breis and the acclimatization temperature of fish between 12° and 37.5° C. The level of oxygen consumption of muscle breis, however, does not differ significantly for fish acclimatized at 12° and 27° C.

3. When measured over the range from 0°.to 37.8° C., the metabolism of fish brain tissue increases rapidly between 0° and 27°, but at higher temperatures the increase with temperature is less marked and at the highest temperature may be lower again.

4. At temperatures up to 27° C., logarithmic plots of the metabolism of brain tissue and of whole animal respiration are similar in slope.

5. Between 4° and 27° C., the rate of opercular movements at the acclimatization temperatures increases with temperature just as does the rate of brain tissue metabolism calculated for the acclimatization temperatures, the ratios of the two being the same at 12°, 20° and 27° C. Above 27° C., however, the rate of the respiratory rhythm does not rise significantly.

6. The rates at which oxygen consumption of the fish, the opercular rhythm and the brain metabolism change during acclimatization to 12° C. after previous ac-

climatization at 27° C. are the same, approximately 50 per cent of the change taking place in two days. This and other observations are interpreted as indicating that the changes in brain metabolism observed to accompany low temperature acclimatization constitute a homeostatic mechanism making possible the maintenance of more normal functioning than would otherwise be possible at the new low temperature.

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REGENERATION IN AN EARTHWORM, EISENIA FOETIDA (SAVIGNY) 1826. III. REGENERATION FROM SIMULTANEOUS ANTERIOR AND POSTERIOR TRANSECTIONS

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This contribution is the third, and last, in a series presenting results of an attempt to obtain, for one particular species of earthworm, complete characterization of regenerative capacity with reference to exact intersegmental levels. In previous parts (1949, 1950) anterior regeneration by posterior substrates and posterior regeneration by anterior substrates were considered. In this part, anterior and posterior regeneration by fragments with two simultaneously made transections is considered.

Summary of Previous Work

Regeneration by earthworm fragments, following two simultaneous transections, has been studied by several of the investigators cited in parts I–II. Operations were usually made at more or less roughly estimated levels, or at approximate levels, sometimes calculated from the posterior end (*i.e.*, from a segment which may be the 125th to the 75th). On first reading, Morgan (1897), for one set of experiments, appeared to have presented results of regeneration, in *E. foetida*, at definite levels. They are summarized, with some rearrangements and omissions, in Table III. That work was done prior to rediscovery of heteromorphosis, in the Oligochaeta, in 1898–1899, since when no attempt has been made, in any Lumbricid species, to determine the morphological nature of simultaneous regenerates, from two transections, that were not quite obviously recognizable as caudal.

Figure 3 is of a fragment "of 14 old segments, two new ones at anterior end and a few new segments also at posterior end. March 20–April 20." Dates are of 13-segment substrates comprising iii-xv. In the figure the substrate at first appears to comprise only 14 segments but use of magnifying glass shows, on the left side, half of a fifteenth segment (unstippled). No 15-segment substrates were mentioned in the table. Both regenerates, as figured, appear to have had metameric abnormalities. Assuming that the drawing of the anterior regenerate shows an attempt, as it were, at formation of a three-segment regenerate, anterior transection may well have been at 3/4, with posterior at 18/19. Otherwise levels may have been 2/3 and 17/18.

Figure 4 is of a fragment "of 13 old segments, and two new ones at anterior end (replacing 3–5) March 20–April 20." The dates mentioned, according to Morgan's Table, were those of 13-segment fragments comprising iii–xv, as well as of 6, 7, and 8-segment fragments comprising "3, 4, 5–10" segments. As the figure shows thirteen substrate segments, anterior transection is taken to have been at 2/3. The

indication in Morgan's Table II that Figures 3 and 4 are of specimens from the "3, 4, 5–10" series is obviously incorrect.

Figure 5, as well as 6 and 7, is, according to Morgan's Table II, of a 14-segment fragment comprising vii–xx. The caption says "13 old segments, and three new ones at anterior end (replacing 7?) April 1–May 13." No 13-segment fragments appear to have had anterior transections at 7/8. Only the first two segments of the figure are stippled (indicative of regenerated segments throughout the plate). The unstippled

TABLE III

Anterior and posterior regeneration, in E. foetida, by fragments from region of i-xxx, according to Morgan (1897 and 1901)

Number of segments	Segments included in substrates	Number of operations	Number of anterior regenerates	Number of posterior regenerates	Morgan's figure no.	Date
3	iii–v	? (a)	+	0		1901 *
4	ii–v	? (a)	+	0		*
8	ii–ix	25	0	0		1897
8	iii–x	50 (b)	1	0 (?)	3, 4 (g)	
10	iv–xiii	10	1	1 (c)		
12	iv–xv	10	1	0		
13	iii–xv	25	2	0		
13	iv–xvi	50 (d)	6	0		
14	vii–xx	55 (e)	8	0 (?)	5, 6, 7 (g)	
16	xv–xxx	26	0	0		
17	iv–xx	15	1	0		
18	iii–xx	26	0	0		
22	ix–xxx	20	0	0		
24	vii–xxx	40 (f)	9	2	8	

According to the text, 81 fragments had the posterior transection at 20/21, but the number according to Morgan's Table was 96.

Omitted above are 25 fragments cut so as to comprise segments "3, 7–15" none of which regenerated, and 25 comprising segments "3, 4, 5–10" two of which regenerated anteriorly (see note b).

* Aside from a statement in a general work, p. 12, no other reference to this experiment has been found.

(a) Number of operations not indicated.

(b) These operations, April 1, according to Morgan's Table, resulted in only one regeneration anteriorly. But the text mentions two pieces, comprising "3–10 segments inclusive" regenerating anteriorly. Presumably one of those was from a March 20 series, omitted from Table above, of 25 animals cut to comprise "3, 4, 5—10" segments. According to Morgan's Table, another fragment of that series also regenerated anteriorly and it could have comprised 6 (v–x), or 7 (iv–x) segments.

(c) A fragment comprising iv–xiii "seemed to have also begun to regenerate at the posterior end" on March 2 (operation Jan. 27) but on March 28 no specimens were found in the pot. On that substrate only, of the 316 (?) with the posterior transection in front of 30/31, was there indication of posterior regeneration, according to Morgan's Table.

(d) Some substrates with regenerates apparently died between examinations.

(e) Of the 25 fragments of March 20 operations, none regenerated. All regenerates were from a second series, of April 1.

(f) Some question as to number of operations on Nov. 9.

(g) In spite of the fact that Morgan s Table and text, except as noted in (c) above, give no indication of posterior regeneration at levels anterior to 30/31, certain figures (camera drawings) and their captions indicate otherwise and accordingly require consideration.

third segment could represent a substrate segment much reduced in size, making 14 substrate segments. Presumably the substrate then comprised segments vii–xx. (Fig. 4 apparently shows an even more marked reduction in size of a substrate segment, in this case the posteriormost.)

Figure 6, according to the caption, is of a fragment of "15 old segments, a few new ones at anterior end (replacing 7) and several new posterior segments." No 15-segment substrates are listed in Morgan's Table. As the figure clearly shows 15 substrate segments, fragments may have comprised segments vii–xxi or vi–xx. The drawing indicates abnormality in the anterior regenerate.

Figure 7 is of a fragment of "13 old segments with five new anterior ones (replacing 7) and a few new posterior segments." The figure shows 14 substrate segments quite clearly, with an unstippled metamere which presumably represents a 15th substrate segment much reduced in size and at first glance apparently part of the posterior regenerate. If the anterior transection was at 6/7 then the posterior transection would have been at 21/22.

Figure 8, according to Table and dates, should be of a 24-segment fragment comprising vii–xxx. According to the caption it is of a 16-segment fragment "with four new anterior segments (replacing 4)" and would then comprise segments v–xx. As the Figure does not show number of segments in fragment, no guess as to original axial location seems feasible. The Figure does show possibility of equimeric regeneration, by a fragment, at an anterior transection as far back as 4/5.

Material, Methods and Nomenclature

In addition to information in corresponding sections of parts I–II (Gates, 1949, 1950) only the following comments are necessary.

Portions of a worm, regardless of size, if with intact buccal or anal region, have been characterized in parts I–II as anterior or posterior substrates respectively. The characterization of "fragment" has been reserved for pieces, regardless of size, with simultaneously made anterior and posterior transections.

Simultaneous, in this article, refers to transections made within a few seconds of each other.

Transections (see Tables IV and V) were made at exactly ten-segment intervals, beginning at 10/11 in one series, at 15/16 in another series; at 15-segment intervals, beginning at 5/6; at 20-segment intervals, beginning, in one series, at 5/6, in another series, at 19/20; and 40-segment intervals, beginning at 35/36. Anterior portions of the first fifteen segments, from one of the series, were transected at 8/9 to give seven-segment fragments, ix–xv, but little or no information was secured from that and other experiments with fragments smaller than ten segments.

Experiments with fragments were conducted from fall to late spring.

In most series, all fragments from the same level, though of different worms, were kept together in one dish. In a few cases, all fragments of one individual were kept together in one dish.

Autotomy, which had been rather annoying in previous work on *Perionyx excavatus* E.Perrier 1872 (Gates, 1927, p. 361) and which may have been an unrecognized source of some of the confusion in Morgan's work on *E. foetida*, was entirely lacking in the author's experiments with anterior and posterior substrates. Two cases

of autotomy, with loss of one and three segments, were observed in the work on fragments. As the loss of even one segment results in a piece of different composition and condition from those originally provided by the operation, fragments that had undergone autotomy were excluded. When operated worms were handled as previously described (Gates, 1949a), rather than being kept in dirt or manure and examined less frequently, recognition of autotomy shortly after the event was possible. No autotomy by regenerates was observed. (In some of Morgan's records of 1902, apparently showing, in examinations at two-month intervals, a temporary decrease in number of segments in regenerates, autotomy or amputation in regenerate presumably was involved.)

Morgan (1897) illustrated in his Figure 17, a fragment "regenerating two posterior ends." Both new growths, drawn as tails, appear to be lateral outgrowths such as may be induced by incisions in the nerve cord. No growths were indicated terminally where transections had healed. It seems preferable to reserve the term *regenerate* for structures replacing removed portions and developing only at level of removal.

Summary of Results

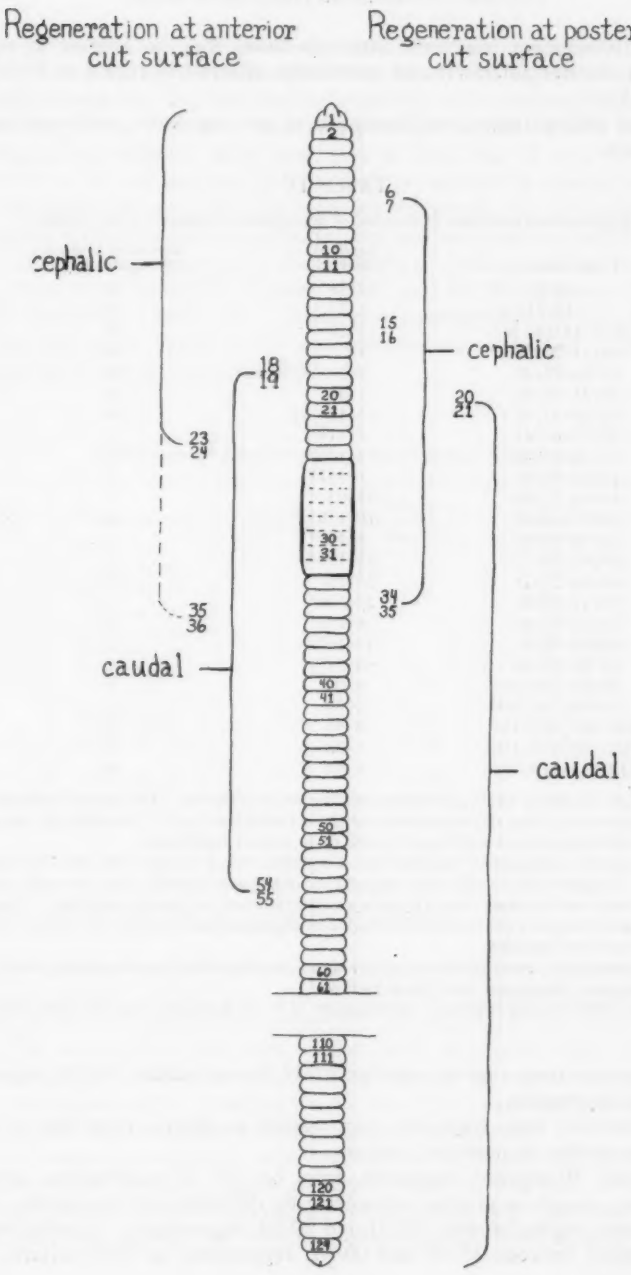
Healing usually was cicatricial. In some fragments the cicatrix, at one or both surfaces, remained imperforate and without externally recognizable change, regardless of lapse of time after operation. In other fragments, at one or both surfaces, and with little or no elevation or protuberance, a cicatrix became characteristically sculptured by formation of a smoothly margined, vertically slit-like aperture that reduced the cicatrix to a narow peripheral band. Often after a variable time, such a sculptured cicatrix slowly became protuberant as a bud, which, with further growth, and metameric differentiation, always became a tail.

Externally recognizable reorganization of substrate segments of the drastic nature later found in *P. excavatus* (Gates, 1941) was not noted. A less drastic form of reorganization involving marked reduction of one or even two substrate segments proximal to the transection, at times to a size only very slightly larger than that of the proximal regenerate segments, was noted in some cases. This type of reorganization involved no change in pigmentation. Some reduction in size charterized all segments of fragments surviving for long periods.

Considerable individual variation as to ability to survive operation and to regenerate was noted. In ten-segment experiments, although three, four, or five fragments of some individuals regenerated, every fragment of other specimens died within the first three days following operation. Several of such individuals were somewhat larger than usual, with especially bright coloration, provided with an unusual amount of chloragogue, and apparently in exceptionally good condition. Fragments of certain individuals survived much longer but without regenerating.

Figure 1. Regeneration along main axis of *Eisenia foetida*. Diagram based on regeneration by substrates with single transection only, and on results of following authors. Anterior regeneration. *Cephalic:* to 23/24, Gates (part I, p. 135); 35/36, author's interpretation of Morgan's estimated levels, 25/26, 30/31, 34/35, and 50/51 (see Table I, part I, pp. 130–131). *Caudal:* at 18/19, Dimon (see part I, p. 135); 20/21–54/55, Gates (part I, p. 135). Posterior regeneration. *Cephalic:* 6/7, author's interpretation of Korschelt's regenerates (see part II, pp. 37 and 43); 34/35, Gates (part II, p. 41). *Caudal:* 20/21, Morgan (see Table II, part II, p. 37), confirmation by Gates (part II, p. 40); no zero level, Gates (part II, p. 41).

Drawing by E. Carpenter, to whom the author's thanks are extended for this assistance.


Regeneration at anterior cut surface
Regeneration at posterior cut surface
cephalic
caudal
cephalic
caudal
1
2
6
7
10
11
15
16
18
19
20
21
20
21
23
24
30
31
34
35
35
36
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41
50
51
54
55
60
61
110
111
120
121
129

FIGURE 1

Survival of 10-segment fragments after operation may be longest in region of 65/66–80/81, shorter anteriorly and posteriorly, shortest in region of 10/11–25/26 (see Table IV).

Segmental differentiation, and formation of new segments, continued long after the first month.

TABLE IV

Regeneration and time of survival of ten-segment fragments of E. foetida

Transections at	Survived, days (a)	Number of fragments that regenerated (b)
(..... 8/9) (c)	2–28	0
(.....10/11) (c)	1–72	0
(8/9–15/16) (c)	1–27	0
10/11–20/21	1–2	0
15/16–25/26	1–8	0
20/21–30/31	1–79	0
25/26–35/36	1–130	0
30/31–40/41	1–118*	3
35/36–45/46	2–128*	4
40/41–50/51	1–127*	4
45/46–55/56	15–93 *	3
50/51–60/61	10–128*	5
55/56–65/66	8–129*	5
60/61–70/71	15–128*	4
65/66–75/76	75–160*	5
70/71–80/81	52–100*	4
75/76–85/86	6–133*	1
80/81–90/91	16–108*	1
85/86–95/96	10–112†	0
90/91–100/101	8–50	0
95/96–105/106	2–94	0
100/101–110/111	8–96	0
105/106–115/116	1–38	0
110/111–120/121	1–94	0

(a) The first figure of each pair indicates day of first death. The second indicates day of death of last survivor or day of preservation of the set indicated by *. A whole set was preserved if substrates with regenerates had begun to die or to appear moribund.

(b) Occasionally a fragment that had had a regenerate was found to be dead at time of daily examination. Regenerates usually had decayed, sometimes so rapidly that the only trace recognizable was a stain on the paper, and at best segmentation was not distinguishable. None of these are included above but all were from within region of regeneration shown in the Table. Sculptured cicatrixes also are not included.

(c) For comparison, records of two sets of short anterior substrates beginning with i and one set of seven-segment fragments have been included.

† Morgan (1902) had a fragment, presumably of 9–15 segments, that was still alive after 7½ months.

All fragments from regions anterior to 30/31 and behind 90/91, regardless of size, failed to regenerate.

All regenerates from fragments were caudal in nature, regardless of whether produced at anterior or posterior surfaces.

Seventy-six 10-segment fragments, from one set of experiments, presumably survived long enough to produce metamerically differentiated regenerates, but only fifty-six, from a region between 30/31 and 90/91, regenerated. Twelve fragments, from the region between 35/36 and 60/61, regenerated at both surfaces, four at

the posterior surface only. Number of segments varied, in homomorphic regenerates, from two to twenty-five when there was no anterior regeneration, from eight to nineteen if there was also anterior regeneration, and in heteromorphic tails from one to fifteen. Total number of segments produced by ten-segment fragments regenerating at both surfaces, varied from nine to thirty-four (1 + 8, 4 + 8, 15 + 15, 15 + 19, etc.). In only one case were numbers of segments in anterior and posterior regenerates equal, and even then the posterior regenerate was obviously the larger. The posterior regenerate always developed first and remained the larger throughout. Occasionally an anterior regenerate did not begin to develop until the second month.

Fragments from the region between 60/61 and 90/91 regenerated only at the posterior transection (Table IV). Absence of regeneration on ten-segment fragments from the region behind 90/91 was independent of position of the fragment, *i.e.*, whether penultimate, antepenultimate, etc.

TABLE V

Regeneration at posterior surfaces by fragments of E. foetida

Number of segments in fragments	Transections at	Number of regenerates*	Number of segments in regenerates	Average number of segments	Average length in millimeters
10	30/31–40/41	3		6.5	
	40/41–50/51	4		13.	
	50/51–60/61	5		7.6	
	60/61–70/71	4		14.	
	70/71–80/81	4		7.	
	80/81–90/91	1	4	4.	1
15	35/36–50/51	8	13–25	20.6	2.8
	50/51–65/66	7	11–21	15.8	2.2
20	35/36–55/56	9	7–27	20.	3.
	39/40–59/60	5	9–18	13.	1.3
	55/56–75/76	6	8–19	11.5	1.5
40	35/36–75/76	6	9–23	14.6	1.5

* Anally sculptured cicatrixes not counted as regenerates.

Of forty-one surviving 15- and 20-segment fragments, forty regenerated only at the posterior surface (Table V). One 15-segment fragment, however, produced anteriorly, at 35/36, a slender heteromorphic tail marked off into three asetal segments. One worm which had been without food for seventy days was cut into 15-segment fragments. Three survived long enough to regenerate but again at posterior transections only (preservation 85 days after operation, period of starvation 155 days). No retardation of regeneration was observed.

Six forty-segment fragments, with transections at 35/36 and 75/76, survived until preservation at 95 days. Each substrate regenerated at both surfaces though anterior regeneration was unrecognizable until some time after posterior growth was quite obvious to the unaided eye. Number of segments in homomorphic tails varied

from 9 to 23 (Table V) and in the always shorter heteromorphic tails from one[1] to eighteen with an average of seven segments, and an average regenerate length of 0.9 mm. Minimum total number of segments in the two regenerates of a single fragment was ten, the maximum, forty-one.

Discussion

A sculptured cicatrix, such as was described above, was not at first considered to be a regenerate. Later on it was discovered that such a cicatrix might grow out into a regenerate that always was caudal and that the aperture of an unprotuberant cicatrix opened into substrate gut. A sculptured but unprotuberant cicatrix accordingly is a small anal region in which a growth zone has not become recognizable and may be considered, even though relatively quite small, a caudal regenerate. For sake of uniformity, the original practice of Morgan was continued throughout. Had anally sculptured cicatrixes been counted as regenerates, percentages of regeneration would, of course, have been higher in the series of this as well as of the two previous contributions. It is probable that an anal regenerate was the structure characterized, prior to rediscovery of heteromorphosis, by Morgan as "not regenerated (mouth present)" (see note f, Table I).

Regenerates, on fragments of ca. 20–22 segments, according to Liebmann (1943, p. 601), showed "all signs of having reached the limit of their growth" at end of one month after operation. The author's results (as well as those from caudal regenerations at single transections, parts I–II) are in agreement with those of Morgan (1902) showing that new segments were produced for several months after operation. When the large regenerative growth zone has been reduced to the very small terminal growth region of normal adult worms, segment formation is quite slow and indications of segment formation may be much more difficult to detect (Gates, 1948).

Absence of cephalic regeneration at anterior transections in region of 30/31–35/36 was unexpected in view of the heteromorphic head obtained at 34/35 (see part II) but is in agreement with the view therein expressed that that level is close to the limit of posterior heteromorphism where head regeneration may be expected only rarely. It is also in accord with the view expressed in part I that Morgan's homomorphic head of five segments at EL50/51 was actually formed at or anterior to 35/36. Failure of fragments from region behind 60/61 to regenerate anteriorly is in agreement with results obtained from normal (unconditioned) posterior substrates which did not regenerate anteriorly at levels behind 54/55.

In a region comprising at least that portion of the axis between 35/36 and 60/61, fragments of ten segments may regenerate at both transections. In view of the ability of forty-segment fragments (35/36–75/76) to regenerate at both transections, it may be expected that fragments of all intermediate sizes also can regenerate at both transections, if from the proper portion of the body, although not observed on the 15- and 20-segment fragments mentioned above.

That portion of the axis behind 60/61, according to the results presented above, comprises two regions; an anterior in which fragments regenerate only posteriorly

[1] An anal region, larger than, and more protuberant than the merely sculptured cicatrix mentioned above, if without indication of segmental differentiation, was counted as a single segment.

and a terminal in which fragments do not regenerate at all. For that portion of the axis in front of 30/31 we are still dependent upon the data of Morgan's experiments discussed in the notes to Table III. Fragments with posterior transection at or in front of 15/16 regenerated at only one transection, the anterior. This suggests possibility of a region with regenerative potentiality the reverse of that prevailing in 60/61–90/91. However, since fragments of as few as three or four segments (at least if comprising iii-v or ii-v) replaced the lost anterior segments, an anterior region, corresponding in reverse to that of no regeneration behind 90/91, would appear to be so small as to be only theoretically recognizable.

In the region behind 60/61, the single regenerate of a fragment is caudal, as in posterior regeneration by anterior substrates. Even in absence of definite information as to the nature of Morgan's single regenerates from the region in front of 15/16, there is no reason for suspecting that they are not cephalic, as in case of anterior regenerates from single transections in that region. This also is the reverse of what takes place in 60/61–90/91.

Some of Morgan's fragments from the region in front of 30/31 had two regenerates. The author's experience with other species, as well as with *E. foetida*, enables a suggestion that the small posterior regenerates shown in two of Morgan's Figures (3 and 7) were cephalic, growth inhibited before completion of metameric differentiation and buccal sculpturing. Such heteromorphic posterior regeneration, at the presumed levels 17/18 or 18/19 (as well as at 21/22) is in agreement with the regenerative potentiality indicated in part II from data of regeneration at single transections. Accordingly there appears to be an anterior region, corresponding in reverse to that of 35/36–60/61, where both regenerates of a fragment are the same, cephalic. The posterior limit of that region at present would appear to be set at 19/20 by the ability of anterior substrates to regenerate a homomorphic tail at 20/21.

The posterior regenerate in Morgan's Figure 6 (1897) is clearly caudal. The posterior transection of that substrate appears to have been at or near 21/22 which is within the region of bipotential regenerative capacity. If wholly within that region fragments such as Korschelt's (1897), according to results of regeneration from single transections, would appear to have the following possibilities; of forming a head and tail at proper anterior and posterior transections, a head at both transections, a tail at both transections, a head posteriorly and a tail anteriorly, a head or a tail anteriorly but no regenerate posteriorly, a head or a tail posteriorly but no regenerate anteriorly. Korschelt's fragments from this region probably had tail regenerates posteriorly. Whether the shorter anterior regenerates were caudal or cephalic was not indicated and how many of the possibilities just listed are actually realizable remains to be determined.

No evidence has yet been discovered to indicate that in *E. foetida* simultaneous regeneration at another transection is able to affect morphological nature of a regenerate, as has been found in an Oriental species (Gates, 1941, pp. 168–171). In this connection may be mentioned the fact that in the former, one regenerate always seems to be much slower in beginning to grow.

The chloragogue of 5–6 segments, in *E. foetida*, is depleted, merely by healing at a single transection, while the chloragogue of 10–15 further segments is required for posterior regeneration, according to Liebmann (1943). Presumably then the chloragogue available in ten-segment fragments should have been consumed merely

by a double cicatrization. Yet as many as 34 segments (in anterior and posterior regenerates together) were produced by a ten-segment fragment. Even at 50/51 and 70/71 ten-segment fragments may produce, on the average, about as many metameres as forty-segment fragments produce at 75/76. These results seem difficult to reconcile with the thesis of proportionality between available chloragogue and segmental constitution of regenerate. A somewhat similar difficulty resulted in Liebmann's suggestion that chloragogue of the regenerating tail provided the balance of material needed. However, regeneration by fragments, after more than two months depletion of chloragogue by starvation, also indicates other sources than chloragogue are available to provide for growth of the regenerates.

Summary

Regenerates from fragments were all caudal and were obtained from a region behind 30/31. Ten-segment fragments regenerated at both transections in the region between 35/36 and 60/61, at posterior transection only in the region between 60/61 and 90/91, not at all behind 90/91; producing as many segments, in homomorphic tails, as 40-segment fragments. The latter, at 35/36–75/76, regenerated at both surfaces. Heteromorphic regenerates developed later, were always smaller, and almost always differentiated fewer segments than homomorphic tails. Starvation did not inhibit regeneration posteriorly by 15-segment fragments.

General Discussion

Dr. Thomas Williams (1851, p. 247) once declared, "On the authority of hundreds of observations laboriously repeated at every season of the year . . . there is not one word of truth" in previous reports of regeneration by earthworms. That, according to Baird (in Johnston, 1865, p. 321) was, "with all due deference, the language of a froward tongue, and unlike that with which a prudent man dealeth with knowledge." However, the Williams dictum did shortly result in further demonstration of the ability of earthworms to regenerate.

It is now known that several species of earthworms may be found in the manure-pile habitat from which *E. foetida* has so often been obtained and evidence is available elsewhere (Gates, 1949b) that some of them at least may have been confused with *E. foetida*. Attention also has been called (note d, Table I) to specific indications that Morgan may have had individuals of such species in some of his experiments.

Nevertheless, when unable to provide confirmation from his own experiments, the present author has followed the practice of accepting provisionally unusual results of others, if they can be fitted into a general pattern. This seems justifiable in view of the fact that individual variation, as to regeneration and morphological nature of regenerate, is possible even in identical experimental conditions, and especially since the same procedures have seldom been used by any two investigators. Some procedural differences may have had more significance than has been realized. Thus, for instance, Liebmann (1942, p. 153) found that *E. foetida* regenerated "incomparably quicker" in earth than in moist paper or linen. However, at 5/6, and in earth, his worms still had, on the twelfth day, only a bud of half-segment width. By that time the author's worms, in moistened paper, already had metamerically differentiated, equimeric, feeding head-regenerates, at least at 2/3 and 3/4.

The anteroposterior axis of *E. foetida,* in recent years, has been demarcated, more or less definitely but variously, according to regenerative capacity. Crowell (1937) marked off at 35/36, two regions: an anterior in which there is no posterior regeneration, and a posterior in which all regeneration, regardless of direction, is caudal. Avel (1937, 1938) recognized three regions: a head regenerating region ending at 20/21, a middle region of no regenerative capacity, and a posterior region in which anterior regeneration, possible only in a limited number of cases, is caudal and heteromorphic. The boundary between the two posterior regions was placed, rather indefinitely, at 35/36 or 40/41 (1937, p. 28), 30/31 or 40/41 (1938, p. 19). Liebmann (1943) recognized but two regions: one anterior to 20/21 in which regeneration is cephalic, and one posteriorly in which regeneration is caudal.

One factor common to each of those schemes is omission of important data long since available, and in particular with reference to posterior heteromorphosis. Such data, together with that provided in the present series of contributions, show that pattern of regenerative capacity in *E. foetida* is more complicated that has hitherto been thought. An attempt to illustrate the pattern diagrammatically has been made in Figure 1. The various boundaries must, however, be understood to be located only provisionally, and based in part on the author's interpretation of results of other investigators.

Reference to Figure 1 will show an anterior region extending back to and including 17/18 in which regeneration, regardless of direction, is cephalic only. In the anterior part of this region, *i.e.,* in front of 6/7, cephalic regeneration seems to be possible only in one direction—anteriorly. In the posterior portion, *i.e.,* from 6/7 to 17/18 inclusive, regeneration may take place in either direction.

The region from 20/21 to 34/35 (± ?) inclusive has a bipotential capacity, as a regenerate, in either direction; may be either a head or a tail. At levels 18/19 and 19/20 regenerative capacity can also be called bipotential. However, present location of two boundaries indicates a one directional restriction on the bipotentiality, *i.e.,* posterior regeneration at 18/19 and 19/20 restricted to heads, but both heads and tails regenerated anteriorily from the same levels.

Behind 34/35 there is another region in which all regeneration is the same, but in this case caudal, and again regardless of direction. This region also can be subdivided into two parts: a more distal portion—posterior to 54/55, in which regeneration apparently takes place in only one direction—posteriorly, and the remaining portion in which a tail may regenerate in either direction. A sort of overall axial symmetry is thus provided by this reversal of regenerative capacity posteriorly. The symmetry is imperfect, however, as the posterior region and both of its sections are much longer than the anterior region and its sections. This greater length apparently provides a section in which fragments theoretically long enough to regenerate actually appear to be unable to do so.

Unless such anteroposterior symmetry is lacking within the region of bipotentiality there should be therein a posterior subregion of one directional restriction the reverse of that apparently characteristic of an anterior subregion comprising 18/19–19/20.

The morphological nature of a regenerate, in *E. foetida,* is controlled by level, according to Crowell (1937); by mesodermal tissues of the ventral half of the body wall according to Avel (1932); by polarized coelomic bodies composed of aggregates of

eleocytes (later called trephocytes) derived from the chloragogue, according to Liebmann (1942–1946). Avel's evidence was provided by several series of unusual and interesting operations but in part from negative results obtained in a region supposedly without regenerative capacity. Liebmann's hypotheses, which have been commented on above and in previous parts, do not explain what organizes the same eleocytes, in one portion of the axis into a head regulating aggregate, and in another portion of the body into a tail regulating aggregate.

In partial agreement with Crowell, level does appear to be of considerable morphogenetic importance. However, bipotential regenerative capacity at numerous levels, in identical external experimental conditions, indicate some further factor or factors should be sought.

Previous discussions of regeneration in earthworms usually have involved an assumption, implied or more or less definitely stated, that pattern of regenerative capacity is identical, not only in species of the same family but even in earthworms in general. Part I showed that the regenerative pattern of the Lumbricid *E. foetida* differs in one very important aspect, inability to regenerate gonads, from patterns of such species of other families as have been studied. Little information as to segmental constitution and morphological nature of regenerates at definite levels is available for other species of the rather small family of the Lumbricidae. Nevertheless, in view of past assumptions as to uniformity of regenerative pattern, certain indications to the contrary are noteworthy.

In *Allolobophora terrestris* (Savigny) 1826, posterior substrates as short as LI4S may regenerate anteriorly (Korschelt, 1897, p. 80). In that species accordingly, the region of anterior heteromorphism now appears to be very much longer, both absolutely and relatively, than in *E. foetida.*

No records have been found of tail regeneration in *Lumbricus terrestris* L. At present it seems necessary to assume that this species is unable to regenerate a tail, at least in the way in which *E. foetida* and many other species do. Yet, on a reversed graft, Ruttloff (1908) obtained a homomorphic tail regenerate at 7/8, a level well in front of any at which there is reason to suspect a tail might regenerate in *E. foetida.*

Segment number may even provide indications as to differences in pattern. Crowell determined boundaries of his morphogenetic regions by data in part from Morgan's experiments on *E. foetida* and in part from his own work on *Allolobophora caliginosa* (Savigny) 1826. His characterizations of regenerative capacity, apparently, were regarded as equally applicable to both species. Recorded maximal segment numbers for those species are 125 and 250, respectively. Accordingly, while level 25/26 has the same numerical value in both species, it is one fifth of the maximum distance toward the posterior end in *E. foetida* but only one tenth in *A. caliginosa.*

In contrast to all such indications is evidence suggesting one contrary trend. In the Glossoscolecid *Criodrilus lacuum* Hoffmeister 1845, Tirala (1912, p. 527) found that regeneration took place "mit Schnelligkeit und Exaktheit" after six months starvation. In the Lumbricid *E. foetida,* as has been shown above and in previous parts, starvation for two or more months does not prevent regeneration. In at least two species of the Megascolecid genus *Perionyx,* the author has found that

starvation of a month and more does not inhibit regeneration. This characteristic alone now seems to be common to the regenerative patterns of different families.

In earthworms, it was long thought that " the exposed surface tends to regenerate all the structures that lie in front of or behind it in the normal worm" (Morgan, 1897, p. 582). Even a small fragment was supposed to have ability to restore all missing segments so as to take again its proper position on the anteroposterior axis (Korschelt, 1897). In *E. foetida* it now appears that a posterior substrate can replace completely the excised portion only if the transection is at or in front of 8/9, although a hypermeric regenerate of nine segments (+ 1, see part I) suggests that further study may enable the level to be placed at 9/10. While head formation at levels behind 13/14 may enable continued existence, reproduction, in absence of ability to regenerate or reorganize gonads, would be impossible.

An anterior substrate can completely reform the excised portion only if the transection is at or behind 20/21 and ordinarily only by a combination of regenerative and normal growth. Presumably then, in *E. foetida*, only fragments having anterior transection at or in front of 8/9 and posterior transection at or behind 20/21 would have any possibility of completely replacing lost portions and again occupying the same location along the anteroposterior axis as before amputation. Other fragments may regenerate but in many cases will be doomed to die of starvation or constipation.

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ADAPTIVE CHANGES IN THE CHLORIDE CELLS OF ANGUILLA ROSTRATA

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The production of hypotonic urine in both marine and fresh water teleosts, together with differences in ion content of the urine which were not accounted for by renal excretion, led Smith (1930) to consider other possible sources of salt elimination. He postulated the gill to be the site of extra-renal chloride excretion. The following year, Keys (1931), through use of a heart-gill perfusion system, demonstrated the excretion of chloride by the gill of the eel, *Anguilla vulgaris*. Histological examination of the epithelium of the eel gill filament by Keys and Willmer (1932) revealed an acidophilic, non-mucous, glandular-appearing cell which they suggested might be responsible for chloride excretion. Recently, Copeland (1948), using *Fundulus heteroclitus*, presented evidence of cytological changes during salinity adaptation. Copeland stated that the presence of a clear vesicle at the free surface of the cell in animals adapted to salt water was indicative of chloride excretion. Pettengill and Copeland (1948) noted the marked difference in alkaline phosphatase activity between sea and fresh water adapted Fundulus and suggested this as possible evidence for the excretory and absorptive functions, respectively.

In the meantime, Krogh (1937), instead of studying excretion as previous investigators had done, measured the ability of several fresh water teleosts to absorb chloride from dilute saline solutions. Of the fish studied by Krogh, the eel was the only species to show no evidence of active absorption.

In extending the study of a possible chloride excreting cell in the gill, it was decided to choose not only another euryhaline teleost, but one which has been the subject of previous histological and physiological investigation. Anguilla fitted these two conditions. The considerable work of Keys and his associates in observing the physiological processes operating in the eel during adaptation, and the interesting observation by Krogh that the eel gill was unique in not absorbing chloride, let to this cytological study of the eel chloride cell during adaptation to fresh and salt water.

Materials and Methods

The experimental work was divided into two sections: a) 4 eels (*Anguilla rostrata*) were adapted to sea water and 3 to fresh water for 3 to 5 days and then killed for a record of the normal picture in those environments; b) 46 eels were divided into two groups and adapted to sea or fresh water for at least 48 hours; they were then transferred to the opposite solution for varying periods—2, 6, 15 or 24 hours—before killing them.

[1] The material reported here is taken from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Arts in Biology in the Graduate School of Brown University.

Part of the animals were experimented upon at Brown University. They were kept in sinks of running tap water or 5 gallon jugs of aerated sea water. The remainder of the eels, investigated at the Marine Biological Laboratory, Woods Hole, Massachusetts, were kept in running sea or tap water aquaria. None of the animals was fed after they were caught.

The eels were killed by beheading and pieces of the gill were fixed for demonstration of Golgi material and mitochondria. The Golgi technique was slightly modified from Ludford's (1926). Fixation was done in Mann-Kopsch solution (2 parts mercuric chloride saturated in 0.5% $CaCl_2$, 1 part 2% osmium tetroxide) for 12–18 hours. The tissue was then washed in distilled water for an hour and postosmicated 3 days at 37° C., followed by an additional day of incubation in distilled water to remove residual tetroxide. When necessary, slides were differentiated in very dilute hydrogen peroxide. For mitochondria the fixation was in Regauds fluid followed by postchromation, then by staining in Altmann's acid fuchsin. The schedule was as described for Fundulus material (Copeland, 1948) except that 1% methlyl green was used for differentiation.

Results

General morphology. The chloride cells of *A. rostrata* are localized in the afferent arterial edge and along the sides of the filaments. They are interspersed among the mucous cells along the edge of the filament and are closely packed together in the filament epithelium between the bases of the respiratory platelets. The latter differs from that in *A. vulgaris*, where the localization is found to extend out on the respiratory platelets (Keys and Willmer, 1932).

The shape of the cells varies with their location. Those at the base of the platelets, where the epithelium is limited in thickness by underlying cartilage, are round

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Figure 1. Sea water adapted 5 days. Mitochondrial preparation in the region of the afferent artery. Note the clefts in the epithelial surface. At the base of one of these clefts a pit typical of sea water adaptation is visible at the free surface of the elongated chloride cell. On either side of it are oval mucous cells.

Figure 2. Sea water adapted 15 hours. Mitochondrial preparation showing cells along the cartilage at the base of a platelet. Compare the shape of these cells with those located along the afferent artery in Figures 1 and 4. These cells are typical of those along the cartilage, which rarely show a pit but often have a clear area of cytoplasm at the surface extending to the supranuclear region. Cartilage is below; section of an adjacent respiratory platelet is above.

Figure 3. Fresh water adaptation 5 days. Mitochondrial preparation in the region of the afferent artery. The chloride cell is less shrunken than those adapted to sea water and the mitochondria are more dispersed.

Figure 4. Sea water adaptation 15 hours. Mitochondrial preparation of the region along the afferent artery. Note the well defined pit and the densely packed mitochondria.

Figure 5. Fresh water adaptation 5 days. Mitochondrial preparation. An example of two fresh water adapted cells along the afferent artery which show definite pits. Compare with Figures 1 and 4 to see that these cells differ from those adapted to sea water in shape and diffuseness of mitochondria.

Figure 6. Sea water adaptation 15 hours. Osmiophilic preparation of the region along the cartilage. At the left can be seen a row of Golgi bodies. At the right, two cells possess varying amounts of granular osmiophilia. In one of them, a band of Golgi bodies is visible just above the nucleus.

Magnification: all Figures to shown scale

FIGURES 1–6

or oval (Fig. 2). The cells in the free edge of the filament are more spindle shaped, often being 3 to 5 times as long as they are wide (Fig. 1). Both variations usually touch the basement membrane and thus are close to blood flowing from the afferent artery to the respiratory platelet.

Completely adapted cell. The cells also vary in shape with adaptation. When sea water adapted, the spindle shaped cells immediately about the afferent artery are quite narrow and irregular in shape (Fig. 1). In fresh water adaptation, they become more plump and oval (Figs. 3 and 5).

In sea water adapted eels, the majority of the long cells adjacent to the afferent artery possess narrow pits (Figs. 1 and 4). Nevertheless, in this species the presence or absence of pits at the distal end of the cells apparently is not an absolute indication of adaptation. In fresh water, although most of the pits disappear, an occasional one can still be seen (Fig. 5). The short, spheroidal cells at the bases of the respiratory platelets rarely show pits under any condition.

The sea water adapted chloride cells possess a dense mass of closely packed mitochondria which fill the entire cytoplasm, except for a sharply delimited crescent-shaped clear area surrounding the pit. In some of the pitted cells this clear region extends as a broad path to a point just above the nucleus (Fig. 4). In the fresh water adapted cell, the mitochondria are more loosely packed.

The osmiophilia varies in amount in both sea and fresh water cells. There is no evidence that it assumes a characteristic form in either adaptation. The Golgi element, which is present in cells adapted to either salinity, usually consists of large granules or bent rods of osmiophilic material arranged in a band across the cell in the supranuclear position (Fig. 6). It is often obscured by varying amounts of a fine granular osmiophilia distributed throughout the cell. The granular osmiophilia, when present, is always evenly distributed throughout the cell, even including the clear areas observed about the pits in Regaud preparations.

Transitional adaptation. Changes in the chloride cell as it adapts to a new salinity are not abrupt. A gradually increasing number of pits is seen in the cells of eels transferred from fresh water to sea water. The cells of animals killed after 2 hours in sea water still have the appearance of those in the fresh water adapted animal, only a few pitted cells being present. The cells of animals stimulated for 6 hours are the first to show a greater abundance of pits. At the end of 15 hours, the filaments appear to be sea water adapted. The chloride cells become more narrow and the mitochondria more densely packed. In the group stimulated for 24 hours, the number of pitted cells does not appear to have increased over that of the 15 hour animals.

The animals transferred to fresh water after 3 days in sea water similarly exhibit gradual changes as they adapt to a lesser salinity. The 2 hours group has the appearance of the sea water adapted animal, but in the 6 hour, and particularly in the 15 and 24 hour animals, there is an evident diminution in the number of pitted cells. A clear area is visible at the free surface in some of the fresh water adapted cells and the mitochondria are less densely packed than in the sea water phase. The animals in the 15 and 24 hour series appear adapted to fresh water.

Discussion

The cytological picture seen in the sea water adapted eel is similar to that found in marine *Fundulus heteroclitus* by Copeland (1948, 1950). Both show a definite structure in the distal end of the sea water adapted cell, the round "excretory vesicle" in Fundulus and the narrow pit in Anguilla. The distribution of mitochondria is similar in the two animals. The amount of osmiophilic material present varies among the cells adapted to a particular salinity and no one picture is apparently typical of the external salinity to which the cell is exposed.

There is not complete agreement in the morphological change during adaptation. When Fundulus adapts to fresh water, the excretory vesicle disappears. This is not paralleled by a complete disappearance of the pit in the chloride cells of Anguilla; instead, an occasional pit persists.

Salt water adaptation. Observations by Keys (1933) on eels and by Smith (1930) on other marine teleosts show that these fish swallow sea water to provide a source of water for the hypotonic urine. The consequent excess of salt in sea water adapted animals is excreted mainly through the gills, and the pits in the chloride cells reflect the operation of an osmotic regulatory process.

The changes in the chloride cell, in animals adapting to sea water, roughly parallel the changes in serum chloride concentration measured by Keys (1933). He notes that movement of water, most of which probably leaves the body by way of the gills, begins as soon as the animal is transferred from fresh to salt water and that chloride is moved across the gills only after a considerable rise in internal concentration occurs as a result of the initial movement of water alone. The present work tends to bear this out. The cells of the 2 hour series appear like those of the fresh water adapted animals and not until the 6 hour series is the quantity of pits typical of the sea water condition begun to be found. It would appear that between the second and sixth hour a threshold had been reached and active excretion begun. Eels of the 15 hour series appear to be completely adapted cytologically, although Keys' experiments show the process is not physiologically complete until about 48 hours.

Fresh water adaptation. Keys' experiments with the eel adapting to fresh water show that water enters the body and that the serum chloride concentration is lowered. He also found, when he used the heart-gill perfusion system, that the gills stop active salt excretion. Cytologically, we find a measurable decrease in the number of chloride cells with pits in animals adapting to fresh water. They never disappear completely, even in the animals which are considered to be completely adapted (3 to 5 days in fresh water). There are no quantitative measurements available to tell whether or not the eel in fresh water continues to transport a small amount of chloride across its gills. The cytological evidence would indicate that possibility.

It is also possible that the presence of a few pits may not be the normal cytological picture, in view of the conditions under which the animals were kept. Grafflin (1931) reported the marked diuresis in marine teleosts kept in live cars, and Meyer (1948) has noted the failure of goldfish to absorb salt from dilute solutions for some hours after the fish were handled. The eels used in this study may have suffered

slight injuries to the skin in the traps or in the somewhat crowded aquaria used for adaptation. In view of the importance which Grafflin and Meyer have shown the immediate environment to have on the physiological state of the animal, a true picture of the chloride cell may not be possible unless bits of gill from animals taken directly from their natural habitat are fixed and observed.

Nevertheless, a distinct difference in the appearance of the chloride cells of eels adapted to fresh or salt water exists. On the basis of the physiological results obtained by Smith and Keys and the cytological picture in Fundulus and Anguilla, the following hypothesis is made. The presence of pits is evidence of chloride excretion in relatively large amounts per cell. Their absence characterizes fresh water adaptation and possible chloride absorption in relatively small amounts per cell. The presence of a few pits in the fresh water Anguilla may indicate a failure to completely adapt to fresh water due to the environmental conditions of the experiment (Grafflin, 1931; Meyer, 1948).

The cytological interpretation of Krogh's physiological observation that Anguilla does not absorb salts in fresh water is not certain. The obvious interpretation of Krogh's results is that salts are not moved at all under fresh water condition. That could be modified by the above hypothesis to the following: Due to different threshold sensitivities of the cells to blood concentrations, a few fail to change to the absorptive condition. The many cells in the absorbing phase are negated by the few cells in the excreting phase, the result being very little net gain or loss.

In conclusion, I wish to thank Dr. D. E. Copeland for suggesting this problem and for his helpful advice and criticism throughout the investigation.

Summary

1. The shape of the chloride cells in the gill of *Anguilla rostrata* varies with their position in the filament. The cells along the free edge have a thin, spindle shape, while those at the bases of the respiratory platelets are more round.

2. Eels adapted to sea water exhibit narrow pits at the free surface of the chloride cells that are located along the afferent artery.

3. The presence of many pits is characteristic of sea water adaptation, but animals adapted to fresh water also possess an occasional pitted cell.

4. The varying number of pits in fresh and sea water adapted animals is correlated with the findings from previous physiological studies of osmotic adaptation.

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A COMPARISON OF NUTRITIVE VALUES AND TASTE THRESHOLDS OF CARBOHYDRATES FOR THE BLOWFLY

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The question has often been raised as to the correspondence between the nutritive value of various substances and their acceptability by animals. Several species of vertebrates and invertebrates have been investigated in this respect. In the rat, for example, Richter (1943) has demonstrated a definite relation between the nutritive value of purified components of various diets and their acceptability. In insects the relation is less definitely established. Only two species have been investigated thoroughly. The results of Vogel's (1931) and von Frisch's (1934) work with the honeybee and Haslinger's (1935) with the fleshfly *Calliphora erythrocephala* showed that substances which are without nutritive value are tasteless but that those with nutritive value may be tasted or not.

Because of the few species of insects tested and the small number of compounds available at that time the status of the question in this class of animals remains in some doubt. Hence, before data from the aforementioned experiments alone become established as a general rule they should be augmented by information derived from studies of additional species and a greater number of compounds.

Few insects are favorable for such combined tests, usually for lack of a suitable technique for measuring sensory responses. Consequently, the nutritional phase of the problem has received the greater share of attention. Bertholf (1927) and Phillips (1927) studied the nutritive value of different compounds for the honeybee; Haslinger (1935) and Fraenkel (1940), for *Calliphora;* Baker *et al.* (1944), for *Anastrepha ludens;* and Hassett (1948), for *Drosophila melanogaster.*

The development of a reliable method for studying the sensory responses of tarsal chemoreceptors by Minnich (1921) and a method for analyzing the data obtained (cf. Bliss, 1938; Dethier and Chadwick, 1948) has opened a way for an extension of the work, begun by Haslinger, on muscoid flies.

The investigation to be described here was divided into two parts: one designed to ascertain the nutritive value of carbohydrates and related substances for the blowfly *Phormia regina* Meigen; the other, an examination of the stimulating effectiveness of these substances for the tarsal chemoreceptors. It is hoped that such an experiment involving large numbers of flies for both phases of the subject will contribute materially toward the solution of the problem.

Methods

The flies used in this work were reared according to the method of Hill, Bell, and Chadwick (1947). For survival tests, pupae were placed in clean jars, and on the day of emergence lots of 100 flies were transferred to 15 × 15 × 15 cm. screen cages. Males and females in approximately equal numbers were used. The cage

front consisted of a sliding glass panel which rested on another glass plate used as a liner for the cage bottom. This bottom plate projected about 2 cm. under the sliding door. On the step thus formed was inverted a vial of the solution to be tested. A strip of filter paper conducted the solution from the vial into the cage and formed a convenient feeding surface. All substances tested were offered in M/10 solution; the vials and paper were changed daily. Dead flies were removed and counted

TABLE I

The survival of blowflies on various compounds, given as summations of daily survival percentages (A), and as days required for 50 per cent mortality (B). M/10 solutions. Each test represents 100 flies

	A	B		A	B
Controls			Disaccharides		
Water, 70% R.H.	212	2½	Maltose	2259	23
Nothing, 70% R.H.	174	2	Sucrose	1573	16
Water, 30% R.H.	120	2	Trehalose	1287	15
Nothing, 30% R.H.	72	1	Melibiose	874	6
			Lactose	339	3½
Pentoses			Cellobiose	264	3½
D-Xylose	367	4½	Trisaccharides		
D-Ribose	343	4			
L-Rhamnose	302	3½	Melezitose	1760	20
D-Lyxose	287	3½	Raffinose	1295	13
L-Arabinose	264	3½			
L-Fucose	227	2½	Alcohols		
D-Arabinose	107	2			
			Sorbitol*	1332	15
Hexoses			Glycerol*	363	4½
			Inositol	299	3½
D Mannose	1914	18	Dulcitol†	243	3
D-Glucose	1636	16½	m-Erythritol‡	247	3
D-Galactose	1636	17	penta-Erythritol‡	204	2½
D-Fructose	1556	14			
L-Sorbose	280	3½	alpha-Methylglucoside§	800	9

* U.S.P., Colgate & Co.
† Eimer and Amend.
‡ Supplied through courtesy of Dept. Agr. Chem., The Ohio State Univ.
§ Eastman Kodak Co.

each day by carefully withdrawing the bottom plate on which they lay. During the experiment the flies were kept in a room at 25° C. and 70 per cent R.H.

Data on survival have been calculated as the number of days from emergence to the time at which 50 per cent remained alive, and also as a score based on the summation of the daily percentage of survival.

The technique employed in testing the stimulating effectiveness of compounds applied to the tarsi of flies has been described fully by Minnich (1929) and Dethier and Chadwick (1947). It makes use of a convenient reflex, the proboscis response. When receptors located on the tarsi are stimulated by solutions of certain substances,

among them being sucrose, glucose, and fructose, the insect responds by extending the retractible proboscis and, when permitted, by drinking. The lowest concentration of a sugar which will elicit this response is termed the acceptance threshold.

TABLE II

The response of Phormia to sugars and polyhydric alcohols

Sugar	Log molar conc. accepted by 50% ±2.575 S.E.	$a \pm$ S.E.*	$b \pm$ S.E.*	$\bar{x}$*	No. of flies tested
Disaccharides					
Sucrose	−2.009 ± 0.319	5.033 ± 0.130	1.045 ± 0.168	−1.978	180
D-Maltose	−2.362 ± 0.631	5.324 ± 0.158	0.750 ± 0.221	−1.931	80
D-Trehalose	−0.875 ± 0.324	4.872 ± 0.148	1.174 ± 0.195	−0.984	120
Cellobiose	20% of the flies accepted at 1.5 molar				60
Lactose	refused in saturated solution by all flies				80
Melibiose	refused in saturated solution by all flies				
Trisaccharides					
Melezitose	−1.196 ± 0.258	4.891 ± 0.147	1.460 ± 0.187	−1.270	120
Raffinose	−0.698 ± 0.131	5.180 ± 0.140	2.791 ± 0.330	−0.633	120
Monosaccharides					
D-Fructose	−2.238 ± 0.131	4.977 ± 0.164	3.252 ± 0.428	−2.245	100
L-Fucose	−1.060 ± 0.245	5.004 ± 0.164	1.734 ± 0.249	−1.058	120
D-Glucose	−0.880 ± 0.227	5.153 ± 0.158	1.782 ± 0.270	−0.794	140
L-Sorbose	−0.854 ± 0.165	4.963 ± 0.164	2.552 ± 0.338	−0.868	100
D-Arabinose	−0.843 ± 0.095	5.429 ± 0.216	3.297 ± 0.675	−0.714	200
L-Xylose	−0.472 ± 0.219	5.120 ± 0.160	1.874 ± 0.329	−0.408	120
D Xylose	−0.357 ± 0.234	5.077 ± 0.148	1.630 ± 0.232	−0.310	120
D-Galactose	−0.302 ± 0.170	4.967 ± 0.148	2.277 ± 0.295	−0.316	120
L-Arabinose	−0.271 ± 0.093	4.832 ± 0.083	2.344 ± 0.202	−0.343	360
D-Mannose	50% of the flies accepted at 7.59 molar				
D-Ribose	0.954 ± 0.752	4.012 ± 0.187	1.530 ± 0.635	0.312	80
D-Lyxose	1.626 ± 1.306	3.708 ± 0.192	1.194 ± 0.513	0.541	100
L-Rhamnose	refused in saturated solution by all flies				
2-Desoxyribose	refused in saturated solution by all flies				
α-Methyl glucoside	−1.159 ± 0.080	5.007 ± 0.114	3.674 ± 0.322	−1.157	240
Inositol	−0.7125 ± 0.160	5.027 ± 0.125	2.012 ± 0.241	−0.699	140

* The 3rd, 4th, and 5th columns of the table give the calculated values for a, b, and $\bar{x}$ in the equation $Y = a + b(X - \bar{x})$, which is the regression of per cent flies accepting, Y, expressed as probits, on log concentration, X. S.E. = standard error.

The sugars and other compounds used in these experiments, with the exception of those noted in Table I, were obtained from Pfanstiehl Chemical Company and were C.P. reagents.

RESULTS

Under the conditions specified for the survival tests, control flies given only water live 2.5 days on the average. If not given water they die somewhat sooner. In a dry (30 per cent R.H.) atmosphere, however, flies survive two days when water is available and only one day when it is not. A humid atmosphere seems to

be more important than available drinking water. These results and those from the feeding tests are shown in Table I.

Since any substance which increases survival materially beyond that of the control flies can be assumed to have some nutritive value, it is evident that all the pentoses tested except fucose are utilized to some extent, with xylose and ribose as the best of a rather poor group. The hexoses are almost uniformly good, sorbose being the single exception. Of the disaccharides, maltose, sucrose, and trehalose are good, the first exceptionally so. Melibiose is intermediate; lactose and cellobiose are poorly utilized. The trisaccharides melezitose and raffinose, and one alcohol, sorbitol, complete the list of well utilized compounds. Alpha-methylglucoside is of moderate value, but the polyhydric alcohols are poor with the exception just noted.

Data showing the acceptance thresholds for *Phormia* are given in Table II. When due consideration is given to the variance of the medians, it is clear that many of the apparent differences in the effectiveness of the various sugars disappear. In general, with this stock of *Phormia* and with these testing methods, a difference in median values to be significant at the 1 per cent level must represent not less than 1½ doubling concentration steps. Under these conditions the effectiveness of the sugars tabulated above is: *disaccharides*—sucrose = maltose > trehalose > cellobiose > lactose; *monosaccharides*—fructose > fucose = glucose = D-arabinose = sorbose > D-xylose = L-xylose = galactose = L-arabinose > mannose = ribose > lyxose.

Discussion

As regards the utilization of various compounds by *Phormia,* a careful examination of the data and a comparison of them with earlier work discloses no significant differences. Minor differences were found such as the utilization by *Phormia* of rhamnose and cellobiose, which other species do not use; but the nutritional value of these substances is so slight that this fly can hardly be said to vary from *Calliphora* or *Drosophila* in its ability to utilize the various carbohydrates. The presence of both an alpha-glucosidase and an alpha-galactosidase is clearly demonstrated. In addition, the tests with lactose and cellobiose indicate a rather low level of activity by a beta-glucosidase and a beta-galactosidase.

Taste thresholds for a large series of carbohydrates have been measured for only seven species of insects. A reëvaluation of the data of Weis (1930) for *Pyrameis atalanta,* of von Frisch (1934) for the honeybee, Haslinger (1935) for *Calliphora,* and Schmidt (1938) for three species of ants shows remarkable similarities to those for *Phormia* when all species are compared (Table III).

In considering Haslinger's results with *Calliphora* it must be remembered that his tests were run with comparatively small numbers of flies, from 20 to 48, and that insufficient data are presented to permit calculation of the variance. It is unlikely, however, that the values obtained with these numbers are any more reliable than those obtained with *Phormia* where from 60 to 360 flies were tested with each sugar. If we may apply the same analysis to Haslinger's data that we have to our own, the following is the order of stimulating effectiveness for the sugars for the legs: *disaccharides*—sucrose = maltose > trehalose = cellobiose > lactose; *monosaccharides*—fructose > fucose > glucose = xylose = galactose = arabinose = mannose; for the mouth *disaccharides*—sucrose = maltose > trehalose = cellobiose >

TABLE III

A comparison of acceptance thresholds (molar concentration) of sugars and polyhydric alcohols for different insects

Compounds	*Phormia* (tarsi) median threshold	*Calliphora* (tarsi mouthparts) starvation threshold (data from Haslinger)		*Apis* (mouthparts) data from v. Frisch	*Pyrameis* (tarsi) medians calculated from Weis' data	*Lasius niger*	*Myrmica rubra*	*Myrmica rubida*
						(mouthparts) minimum threshold (data from Schmidt)		
Sucrose	0.0098	0.0006	0.0035	0.0625–0.125	0.02	0.005	0.0066	0.0025–0.00125
D-Maltose	0.0043	0.00125	0.002	0.0125	0.45	0.01	0.01	0.01
D-Trehalose	0.133	0.14	0.02	0.25	>1.0	—	—	—
Lactose	*	*	0.1	*	*	*	*	*
Cellobiose	5.01	0.05	0.03	*	>1.0	*	—	*
Melibiose	*	*	*	*	—	—	*	*
Melezitose	0.064	0.07	0.01	0.125–0.25	0.93	0.01	0.005	0.0156
Raffinose	0.20	0.10	0.007	*	0.45	0.0025	0.0025	0.01
D-Fructose	0.0058	0.0033	0.004	0.25	0.15	0.0156	0.02	0.03125
L-Fucose	0.087	0.02	0.01	1.0	0.03	—	—	—
D-Glucose	0.132	0.125	0.04	0.25	0.20	0.125	0.02	0.03125
L-Sorbose	0.140	—	—	—	—	—	—	—
D-Arabinose	0.144	—	—	*	—	—	—	—
L-Xylose	0.337	—	—	—	—	—	—	—
D-Xylose	0.440	0.2	0.14	*	>1.0	—	—	*
D-Galactose	0.50	0.14	0.09	2.0	>1.0	*	0.5	*
L-Arabinose	0.536	0.1	0.08	*	0.85	*	*	*
D-Mannose	7.59	0.2	0.1	*	*	*	0.25	*
D-Ribose	8.99	—	—	—	—	—	—	—
D-Lyxose	42.27	—	—	—	—	—	—	—
2-Desoxyribose	*	—	—	—	—	—	—	—
L-Rhamnose	*	*	1.0	*	—	*	—	*
α-Methyl glucoside	0.069	0.01	0.03	0.50	0.20	0.125	0.02	0.125
Inositol	0.194	0.03	0.03	ca 0.25	*	—	—	—
Glycerol	*	—	—	—	—	—	—	—
Sorbitol	*	0.03	0.08	*	—	0.25	0.0625	*
Dulcitol	*	0.03	0.08	*	>1.0	*	*	*
Mannitol	*	0.1	0.14	*	>1.0	*	1.0	*
M-erythritol	*	—	—	*	*	*	*	*
Penta-erythritol	*	—	—	—	—	—	—	—

* Non-stimulating at all concentrations. —Not available for testing.

lactose; *monosaccharides* — fructose ≧ fucose > glucose ≧ arabinose = xylose = galactose = mannose. A similar treatment of Weis' data gives the following order: *disaccharides*—sucrose > maltose > trehalose? cellobiose > lactose; *monosaccharides*—fucose > fructose > glucose > L-arabinose > xylose? galactose > mannose. Schmidt's data are in the order: *disaccharides*—sucrose ≧ maltose > cellobiose; *monosaccharides*—fructose ≧ glucose > galactose. Von Frisch worked with considerably larger numbers of bees in his field experiments. For disaccharides the order of effectiveness is: sucrose = maltose > trehalose; for *monosaccharides,* fructose = glucose > fucose > galactose. All other sugars which were tested with bees failed to stimulate in saturated solution.

Considered thus, the outstanding features of the eight sets of threshold data are the similarities rather than the differences (Table III). The three outstanding discrepancies are the value of cellobiose, which is considerably stimulating to *Calliphora;* the value of fucose, which to the bee is less stimulating than glucose and to *Pyrameis* is more stimulating than fructose; the value of raffinose, which is very low for the mouthparts of *Calliphora* and for ants. The fact is inescapable, however, that the most stimulating disaccharides are those which contain glucose or

TABLE IV

A comparison of the nutritive value of carbohydrates and related compounds with the acceptance thresholds for Phormia

Compound	Comparative nutritional value	Molar concentration at threshold
D-Maltose	++	0.0043
D-Fructose	++	0.0058
Sucrose	++	0.0098
Melezitose	++	0.064
α-Methylglucoside	++	0.069
L-Fucose	−	0.087
D-Glucose	++	0.132
L-Sorbose	−	0.140
D-Arabinose	−	0.144
Inositol	+	0.194
Raffinose	++	0.200
L-Xylose	+	0.337
D-Xylose	+	0.440
D-Galactose	++	0.500
L-Arabinose	+	0.536
Cellobiose	+	5.01
D-Mannose	++	7.59
D-Ribose	+	8.99
D-Lyxose	+	42.27
Melibiose	++	*
Sorbitol	++	*
Glycerol	+	*
Dulcitol	+	*
Meso-Erythritol	+	*
Penta-Erythritol	−	*

fructose units and can be hydrolized by alpha glucosidases, and the most stimulating monosaccharides are fructose, glucose, and fucose. The polyhydric alcohols are, in general, ineffective, but inositol is appreciably stimulating to *Phormia* and *Calliphora.* Further comparisons are impossible because of the lack of uniformity of testing methods employed by different authors. It should be noted that each reports different criteria of stimulating effectiveness.

The data in hand from both phases of this study indicate that it is not possible to correlate the taste of a substance and its nutritive value, as Vogel was able to do to some extent. Her data for the honeybee show that all sweet substances are utilized while both nutritious and non-nutritious substances are included in the tasteless group. The present study reveals, on the contrary, that all gradations of taste

thresholds occur for nutritious and non-nutritious substances. If these materials be arranged in order of their threshold values (Table IV), most of the substances which are well utilized are found among those with low threshold values, but mannose, at the top nutritionally, has a threshold far above the level it might be expected to occupy; conversely, fucose, with zero value as food, is very stimulating.

On the basis of all studies to date it would appear that with some insects at least there is no fixed correlation between the nutritive value of substances and their acceptability.

Summary

Thirty sugars and related compounds have been tested on the blowfly *Phormia regina* Meigen to determine their nutritive value for this species and their effectiveness as stimuli for the tarsal receptors.

Survival tests indicated that all pentoses except fucose are utilized to some extent. Xylose and ribose are the best. The hexoses are almost uniformly good. Sorbose is the exception. Maltose is the most effectively utilized disaccharide. Sucrose and trehalose are good; melibiose, intermediate; lactose and cellobiose, poor. The trisaccharides melezitose and raffinose and the alcohol sorbitol are effective. In general the polyhydric alcohols are poor.

The stimulating effectiveness of the sugars when applied to the tarsi is as follows: *disaccharides*—sucrose = maltose > trehalose > cellobiose > lactose; *monosaccharides*—fructose > fucose = glucose = D-arabinose = sorbose > D-xylose = L-xylose = galactose = L-arabinose > mannose = ribose > lyxose.

There is no good correlation between the nutritive value of these compounds and their acceptability.

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STUDIES IN THE REGULATION OF BLOOD-SUGAR CONCENTRATION IN CRUSTACEANS. II. EXPERIMENTAL HYPERGLYCEMIA AND THE REGULATORY MECHANISMS [1]

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In the first paper of this series (Kleinholz and Little, 1949), the literature of hyperglycemia in crustaceans was reviewed and some experimental conditions effecting increase in total reducing substances of crustacean blood were examined. This increase in total reducing substances of the blood was shown to be in the fermentable component, and therefore probably represented a true hyperglycemia. The wide range of these glycemic values, sometimes even in the same species studied by different investigators, soon led to recognition of the need of maintaining animals under known laboratory conditions, and to the employment of standard analytical methods. With these precautions, experimental modification of the glycemic level in crustacea became more reliable. The studies of Hemmingsen (1924), later extended by Lindblad (1931), on the rate of disappearance of injected glucose from the blood of Astacus, indicated the possible presence of a hypoglycemic regulatory mechanism. Little attempt has been made to localize this mechanism anatomically.

A group of subsequent studies by various investigators, probably influenced by the known glycemic effects of adrenaline and insulin among vertebrates, reported results of injecting these substances into crustaceans. Some of the results were contradictory and for a time discussion centered not so much about the interpretation of the reported experimental results as about the specificity of the particular pharmacological agent in producing its effect. Thus, Roche and Dumazert (1935) stated that neither insulin nor adrenaline had any effect on the blood-sugar concentration of *Cancer pagurus*. Medvedeva (1936) found that injection of adrenaline in Potamobius (syn. Astacus) caused hyperglycemia, but that injection of insulin had no definite effect. Kalmus and Waldes (1936), questioning the specificity of the adrenaline-induced hyperglycemia, observed that not only adrenaline and insulin, but also such non-specific substances as hydroquinone and sodium chloride resulted in marked increases in blood-sugar concentration when injected into crayfish. Florkin and Duchateau (1939) attempted to resolve some of these contradictions by more carefully controlled procedures, and reported that insulin had no effect while adrenaline produced hyperglycemia in crayfish, thus confirming the earlier observations of Medvedeva.

These observations, questionable as may have been the interpretation of the specificity of the pharmacologcial effect, indicated the possible existence of a hyperglycemic mechanism (as Hemmingsen's studies had indicated a *hypoglycemic*

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mechanism). Abramowitz, Hisaw and Papandrea (1944) demonstrated that a definite anatomical structure might be involved in this hyperglycemic mechanism. These investigators found that injection of aqueous extracts of crustacean eyestalks into *Callinectes sapidus* brought about a marked increase in blood-glucose concentration; more specific localization showed the source of this hyperglycemic or diabetogenic hormone to be associated with the sinus gland within the eyestalk, a structure that has already been demonstrated to participate in a number of endocrine processes in crustacea (Kleinholz, 1942; Brown, 1948).

Our own interest in investigating the nature of this hyperglycemic mechanism was stimulated by indications in the literature, not further explored, of hyperglycemic responses in crustaceans similar to the excitement hyperglycemia reported in mammals. Stott (1932) had reported a large increase in blood-sugar concentration of several crustaceans after asphyxiation, obtained by keeping them for 10 hours in tightly-covered containers filled with sea water. Roche and Dumazert (1935) confirmed this observation on *Cancer pagurus;* they found that removing animals from sea water and keeping them in air for 30 to 60 minutes resulted in marked hyperglycemia. Both studies reported this increase in glycemic level as a direct observation without investigating in further detail the mechanism of this response. In the first paper of this series (Kleinholz and Little, 1949) similar results were obtained with Libinia, and a possible mechanism for this hyperglycemia was indicated by the fact that animals from which both eyestalks had been removed no longer showed great increases in blood-sugar after asphyxia.

The next logical and traditional development, after injection of extracts, in proving hormonal participation of sinus gland in blood-sugar regulation, would be actual demonstration of the mediation of the sinus gland in experimentally-produced hyperglycemia. Removal of sinus glands by ablation of eyestalks, as was done with Libinia, is not sufficiently critical to be considered conclusive proof of such mediation for reasons which are explained below in the next section on methods. Surgical removal of the sinus gland from the eyestalk of Libinia is difficult because of the small size of the stalk and its lesser accessibiltiy than in other crustaceans. More exact studies in which sinus glands were removed with little damage to the rest of the eyestalk were postponed until the time when surgically more convenient animals like Astacus and Callinectes could be used.

The present report describes experiments which prove conclusively the mediation of the sinus gland in cases of physiologically-induced hyperglycemia, and which investigate the resemblance between experimental hyperglycemia in crustaceans and the excitement hyperglycemia of higher vertebrates.

Materials and Methods

The animals used in this study were the common Oregon crayfish, *Astacus trowbridgii,* and the blue crab of the Atlantic Coast, *Callinectes sapidus.* Crayfish were maintained in the laboratory in tanks through which tap water circulated, a depth of about two inches of water being maintained. Each of these tanks was covered to provide shaded areas, while the tanks themselves were located in an unheated, well-ventilated room. A supply of Callinectes was kept in a large outdoor concrete tank supplied with running sea water.

Stock crayfish were fed approximately once a week on freshly-killed frogs, clams, or bits of beef. The storage tank in which Callinectes were kept had an

abundance of food material in the form of freshly-killed fish and discarded crabs which the living crabs readily scavenged. The individuals constituting some of the early experimental groups were isolated and starved for 3 to 5 days before undertaking any experimental procedure. In later groups individuals were isolated for no more than a day because the observations reported here are concerned chiefly with relative changes in a period of a few hours rather than with absolute changes in the concentration of blood-sugar.

At least one day before taking the first blood sample the carapace of each crayfish was punctured with a dissecting needle in the mid-dorsal line directly over the pericardial cavity, and the hole then plugged with a bit of modelling clay. In taking blood samples from Astacus this plug was removed and about 0.15 ml. of blood taken by means of a 20-gauge hypodermic needle fitted to a graduated 1 ml. tuberculin syringe. The opening into the pericardial cavity was re-sealed with modelling clay, and the animal returned either to water or to its particular experimental environment. In Callinectes blood samples were taken by puncturing the arthrodial membrane at the base of one of the walking legs with the needle of the hypodermic syringe. An exact 0.10 ml. blood sample was transferred from the syringe to a calibrated serological pipette. The blood was then blown into a tube containing acid cadmium sulfate and rinsed half a dozen times with this deproteinizing mixture. Blood samples, both under control and experimental conditions (except where asphyxia of Astacus was attempted by prolonged maintenance in air), were always taken in the fore-noon to avoid the possibility of diurnal and nocturnal fluctuations in the concentration of blood-sugar. Male individuals only were used in these studies, the crayfish ranging from about 80 to 100 grams and the crabs from about 120 to 150 grams in weight.

Groups of six animals were used at a time. In all cases blood samples were taken from the individuals of a group before a particular experimental treatment and then a second time immediately after such treatment. In this way each animal served as its own control to the experimental condition used. Both the control and the experimental blood samples received comparable handling, being carried through the analytical procedure simultaneously. The method of Miller and Van Slyke (1936) was used in determining the blood-sugar concentration of the samples. Three blanks were prepared with each set of control and experimental blood samples. These blanks required from 0.13 to 0.28 ml. of the dilute ceric sulfate to attain the same end-point reached in the titration of the blood samples. The average volume of this reagent required for 120 such blanks was 0.184 ml.; this compares with an average of 0.195 ml. for 58 similar blanks in the preceding study of this series (Kleinholz and Little, 1949). No distinction was attempted between fermentable and non-fermentable components of the blood, so that the values reported in this study represent the total reducing substances expressed as glucose equivalents.

Effective asphyxia which had been obtained with Libinia by removing the animal from water to an air environment was similarly accomplished with Callinectes. This method, however, was not successful with Astacus. Asphyxia was therefore induced by immersing these animals for stated periods in de-aerated water. A heavy-walled flask containing 2 liters of water was de-aerated by connecting the flask to a vacuum pump and maintaining the reduced pressure for 15 minutes after

vigorous bubbling had commenced. At the end of this time the vacuum was broken and the water decanted into screw-top jars of about 500 ml. capacity. Each jar was filled to the brim and a crayfish lowered into it, the displaced water overflowing the jar; the screw-top, fitted with a rubber gasket, was applied to exclude air bubbles and tightened. At the end of the period of asphyxia the animal was removed from the jar and the second blood sample taken. Crayfish were usually limp after this asphyxia, but recovered rapidly after being returned to their tanks.

The effect of chloroform anesthesia on concentration of blood-sugar was studied by immersing crayfish for various periods of time in aqueous solutions of chloroform. The solutions were prepared by vigorously shaking small measured volumes of chloroform with 500 to 1000 ml. of water and then diluting with additional volumes of water to give the desired concentrations. Enough solution was prepared and poured into a small tank so that the surface was about one-half inch above the dorsal carapace of the immersed individual. After the periods of immersion the animals were removed and blood samples taken for the second time before the crayfish were returned to their stock tanks for recovery.

Study of the effect of adrenaline on the concentration of blood-sugar was made by injecting dilutions (in distilled water for Astacus and in 0.7% saline for Callinectes) of Parke Davis & Company "Adrenalin (1:1000)" into the blood sinus at the base of a walking leg. These dilutions and the volume injected (0.1 to 0.2 ml.) were arranged to give a series ranging from 150 γ to 0.1 γ. Comparable volumes of distilled water, of 0.7% saline, and of saturated aqueous chloretone were used as control injections. The dilutions were prepared on each occasion of use from 1 ml. ampoules. In this group of experiments each animal served as its own control, the procedure being to take the first set of blood samples before injecting the desired solution and then, 90 minutes after the injection, to take the second blood sample.

The role of the sinus gland in the hyperglycemic responses obtained by the various procedures described above was determined by repeating these procedures on animals from which the sinus glands had been removed. In many early studies removal of the sinus gland was effected by simple ablation of both eyestalks, this procedure being frequently dictated by the difficulties of surgical operation on very small eyestalks. Such investigations could be criticized on the basis that ablation of entire eyestalks removed simultaneously, in addition to the sinus glands, the four large ganglionic nerve masses in each stalk, as well as the so-called X-organ which has been suspected of secretory function. Removal of the sinus gland alone is now feasible by methods which have been described by Brown (1942), by Panouse (1946) and by Kleinholz (1947). The last-named method by which the sinus gland and its large nerve can be readily exposed was used in excising the gland with little or no damage to the optic ganglia, and also to denervate the gland.

Results of Asphyxia

In the first of these studies (Kleinholz and Little, 1949), it was reported that asphyxia in Libinia, obtained by removing the animals from sea water and exposing them to air for an hour, caused a marked hyperglycemia, the resulting concentration of blood-sugar being 2 to 3 times that before the asphyxiating treatment. The earlier-mentioned report of Abramowitz, Hisaw and Papandrea (1944) suggested

to us the possibility that the sinus gland might be concerned in the regulation of blood-sugar concentration. Exploration of the possibility in Libinia added further credence to this view when it was found that individuals from which both sinus glands had been removed by excision of the eyestalks no longer showed the marked hyperglycemia of asphyxia demonstrated by normal individuals. This evidence could not, however, be considered as conclusively demonstrating that the sinus gland was responsible for the glycemic effect because, as has been explained above, eyestalk ablation removes a considerable amount of other tissue. The effect of asphyxia was, therefore, re-examined in the present study in normal animals and in animals in which only the sinus glands had been excised or denervated.

TABLE I

The effect of asphyxia on the concentration of blood-sugar

The concentration of blood-sugar before and after asphyxial treatment is given in milligrams per 100 ml. of blood (mg.%), with the standard deviation from the average of the group; the numerals in parentheses following the concentration after asphyxia indicate the number of animals in the group that showed increases of less than 3 mg.%; *A*, asphyxia attempted by keeping the animals in air; *—ES*, eyestalkless animals; *—SG*, sinus-glandless animals; *—SGN*, animals in which both sinus glands had been denervated; *W*, asphyxia accomplished by maintaining animals in de-aerated water.

Animals		Asphyxia		Average mg.% blood-sugar		Average per cent increase
Condition	No. used	Method	Duration	Before	After	
Astacus:						
Normal	5	A.	2 hrs.	16.5 ± 9.5	15.6 ± 8.1 (4)	0
Normal	5	A.	4 hrs.	19.7 ± 2.4	19.1 ± 2.4 (5)	0
Normal	5	A.	8.5 hrs.	20.0 ± 3.8	18.7 ± 5.5 (5)	0
Normal	10	W.	1 hr.	16.7 ± 7.9	29.0 ± 21.1 (4)	74
—SG	9	W.	1 hr.	14.3 ± 8.9	14.2 ± 8.7 (8)	0
Normal	16	W.	3 hrs.	18.3 ± 6.5	52.9 ± 22.4 (1)	189
—SG	10	W.	3 hrs.	11.6 ± 5.8	12.7 ± 5.1 (8)	9
Callinectes:						
Normal	4	A.	2 hrs.	9.2 ± 1.7	27.7 ± 10.3 (0)	201
—ES	6	A.	2 hrs.	9.7 ± 3.8	8.0 ± 3.2 (6)	0
—SGN	11	A.	2 hrs.	14.4 ± 4.9	16.9 ± 6.5 (6)	17

Attempts to reproduce the hyperglycemia of asphyxia in Astacus by removing the animals from water and exposing them to air, in much the same manner as had been employed with Libinia, were unsuccessful. No appreciable change in concentration of blood-sugar was obtained with Astacus which had been kept for 2, 4, and 8.5 hours in air (Table I); in 5 of the 15 animals very slight rises occurred after treatment, two animals showing increases of 4.3 and 4.0 mg.%, while the three other increases ranged between 0.5 and 2.0 mg.%; in the 10 remaining animals small decreases in blood-sugar values, from 0.6 to 5.5 mg.%, were observed.

A more effective method of asphyxia was found by immersing individuals in water from which air had previously been removed by vacuum. When normal Astacus were immersed for 1 hour in such de-aerated water, the effect on the concentration of sugar in the blood was variable: 4 of the 10 treated animals in Table I

showed very marked increases, the blood-sugar concentration being 2 to 3½ times the concentration before asphyxia was begun; 2 crayfish showed lesser rises, while the remaining 4 individuals of this group showed small decreases in concentration ranging from 0.8 to 2.7 mg.%. The average increase for this group as a result of asphyxia was 74 per cent. When 9 Astacus from which the sinus glands had been removed were tested in the same way, the group average for blood-sugar concentration after asphyxia was not significantly different from the average before treatment. In 2 of these animals there was a post-asphyxial increase in blood-sugar concentration amounting to 5.7 and 1.7 mg.%, while in 6 animals slight decreases ranging from 0.4 to 2.3 mg.% occurred.

To see whether more uniform hyperglycemic responses could be obtained, groups of normal crayfish were asphyxiated as before, but the exposure was increased to 3 hours. Of 16 animals thus treated, 15 showed post-asphyxial concentrations of blood-sugar that were from 1½ to 6 times greater than the pre-treatment glycemic values, while one animal showed a decrease of 5.4 mg.%. The average increase as a result of asphyxia was 189 per cent. Ten sinus-glandless Astacus which were exposed for 3 hours to the same anoxic conditions showed an average rise of 1.1 mg.% in blood-sugar concentration. Of these 10 animals the distribution of change was as follows: 2 showed increases of 7.7 and 8.2 mg.%, 3 showed slight increases between 1.0 and 2.0 mg.%, while 5 showed slight decreases ranging from 1.0 to 2.7 mg.%.

With Callinectes the hyperglycemia of asphyxia could be obtained by using the same method employed with Libinia, *i.e.*, removing the animals from a water to an air environment. Blood-sugar concentrations in normal crabs after 2 hours in air were 2 to 4 times the pre-asphyxial level, with an average increase of 201 per cent. No hyperglycemia occurred in 6 Callinectes which were asphyxiated after ablation of both eyestalks; 5 of these animals showed insignificant decreases in glycemic level, the maximum being 1.2 mg.%; the average decrease of 18 per cent for the group is due to a very marked drop of 8.2 mg.% in the sixth animal.

An experiment of additional interest was performed with Callinectes in which the relatively large sinus gland nerves were cut. In 2 animals bilateral denervation of the gland was done 11 days before and in the remaining 9 animals from 12 to 36 hours before the beginning of the experiment. The group average for blood-sugar concentration after asphyxia showed an increase of 17 per cent, in striking contrast to the 201 per cent increase obtained with normal individuals after similar treatment. Whether this 17 per cent increase is significant, in view of the relatively small number of animals used in the experiment, is difficult to determine. Four individuals of this group which had been operated 11 days, 36 hours, 16 hours, and 12 hours before beginning the experiment showed increases in the glycemic concentration respectively of 1.8, 0.6, 0.6, and 2.7 mg.%; 5 Callinectes showing somewhat larger increases, 8.0, 7.0, 6.4, 3.5, and 3.1 mg.% had been operated, respectively, 16, 14, 23, 18, and 21 hours earlier; 2 crabs which showed decreases of 2.3 and 3.6 mg.% had been 11 days and 12 hours, respectively, since the operation of denervation. There thus seems to be no definite relation between the recency of surgical procedure and the slight hyperglycemia produced by asphyxia in the animals with denervated sinus glands. Denervation of this gland prevents the considerable hyperglycemia that appears in asphyxiated normal animals and indicates the partici-

pation of the central nervous system in producing this effect. A possible explanation of the slight rise in glycemic level in animals with denervated sinus glands will be considered below in the discussion of the possibility of sources of hormone outside these glands.

Results of Anesthesia

The readiness with which crustaceans responded by a rise in blood-sugar concentration to crowding and handling (Abramowitz, Hisaw and Papandrea, 1944), and to asphyxia (these studies), led us to suspect that this response might be analogous with the excitement hyperglycemia of mammals. Since a number of investigators have reported that surgical anesthesia in mammals produces marked hyperglycemia, we sought to determine whether chloroform anesthesia would similarly effect an increase in the blood-sugar concentration of Astacus.

Table II

Effect of chloroform anesthesia on blood-sugar concentration in Astacus

Animals		Anesthesia		Average mg.% blood-sugar		Average per cent increase
Condition	No. used	Concentration	Duration	Before	After	
Normal	11	1:1750	1 hr.	15.9 ± 4.1	21.6 ± 5.2 (3)	36
Normal	11	1:1750	1 hr.	36.2 ± 10.8	67.5 ± 12.4 (0)	86
—SG	16	1:1750	1 hr.	16.0 ± 5.6	16.6 ± 5.2 (11)	4
Normal	18	1:830	1.5 hrs.	24.0 ± 8.8	38.0 ± 8.6 (3)	58
—SG	7	1:830	1.5 hrs.	12.2 ± 5.7	12.3 ± 8.3 (5)	1
Normal	17	1:830	2.0 hrs.	24.1 ± 7.3	72.5 ± 28.2 (0)	200

The necessary concentration and duration of treatment with chloroform were worked out by trial from the known experience (Kleinholz, 1947) that exposure of the crayfish, Cambarus, for 2 to 3 minutes to a concentration of approximately 1 : 500 of chloroform in water was adequate for surgical anesthesia. With less concentrated solutions we succeeded in anesthetizing animals and maintaining them in this state for 1 to 2 hours with complete recovery following their return to flowing water. The effects of such anesthesia on the concentration of blood-sugar in normal Astacus and in individuals from which both sinus glands had been removed are summarized in Table II.

In the first group, with a concentration of 1 : 1750, 3 of the 11 animals tested showed little increase in glycemic level, while the remaining 8 Astacus showed individual increases in blood-sugar concentration ranging from 4.8 to 10.7 mg.% as a result of the chloroform treatment. The average increase of 36 per cent, while marked, is not as striking as the increases obtained by asphyxia in the preceding section.

A second group of 11 crayfish differed from the first group in that the animals had not been starved before the experiment; as a result the concentrations of blood-sugar were relatively high. This group demonstrated a more striking hyperglycemia after anesthesia, even when treated in the same manner as the first group with respect to duration of exposure and concentration of the chloroform solution. The individual increase in these after anesthesia was not less than 12

mg.%, the glycemic levels ranging from 1½ to 3 times those before anesthesia; the average increase of 86 per cent is considerably higher than that obtained with the first of these groups.

The difference in hyperglycemic response between the two similarly treated groups may be due to a greater carbohydrate reserve in the case of the fed animals, thus making possible a greater rise in glycemic concentration, or it may be due to individual differences in sensitivity to chloroform. The possibility of this latter factor is indicated by the differences in the degree of hyperglycemia shown by two other groups of normal Astacus which were immersed in more concentrated chloroform solution (1:830). In one group of 18 Astacus the average increase was 14 mg.%. In the second group consisting of 17 individuals treated with the same concentration of chloroform, and having an average blood-sugar concentration before anesthesia practically identical with the preceding group, an increase of 30 minutes in the time of exposure to chloroform resulted in a much more striking hyperglycemia. All the individuals showed glycemic values that were from 1½ to 9 times the concentration before treatment; the average increase in this second group was 200 per cent, compared with the 58 per cent average increase shown by the former group. The degree of hyperglycemia may thus depend on the concentration of the anesthetic and the duration of exposure to it.

The effect of chloroform anesthesia on animals from which both sinus glands had been removed was quite different from the responses of the normal crayfish. When 16 sinus-glandless animals were exposed for 1 hour to 1:1750 chloroform, 8 showed either no change or the minor fluctuations of plus or minus 1 mg.%, 5 showed an increase in blood-sugar concentration ranging from 3.0 to 6.4 mg.%, and in the remaining 3 decreases of 4 to 5 mg.% occurred. The average concentration of blood-sugar after chloroform treatment was only 4 per cent higher than the average concentration before treatment, as compared with the glycemic increases of 36 per cent and 86 per cent shown by normal animals under similar conditions.

A second group of 7 sinus-glandless animals was similarly tested with the higher concentration of chloroform water (1:830) for one and one-half hours. Two crayfish showed increases of 3.1 and 5.6 mg.%, three showed slight decreases in glycemic level ranging between 2.0 and 4.6 mg.%, while the remaining two individuals showed no change. The average glycemic level after anesthesia was less than 1% higher than the initial concentration of blood-sugar.

It may thus be concluded that the presence of the sinus glands is necessary for the marked hyperglycemia of anesthesia to occur.

Results of Adrenaline Injection

We decided to re-study the effect of adrenaline on glycemic level in crustaceans, not so much for the analogy with its hyperglycemic effect on vertebrates, but because the sinus gland is prominently innervated. Hanström (1937) and Welsh (1941) have described the sinus gland nerve as originating in the large optic ganglion, the medulla terminalis; in Cambarus, Welsh has found additional fibers originating in the supra-esophageal ganglion which join those from the medulla terminalis to constitute the sinus gland nerve. Evidence from our experiments in Callinectes whose sinus gland nerves had been transsected, indicated that the hyperglycemia of asphyxia was mediated through stimuli that passed to the gland by way of this nerve. It

TABLE III

Effect of injection of adrenaline and of control substances on the concentration of blood-sugar

Animals		Injection		Average mg.-% blood-sugar		Average per cent increase
Condition	No. used	Substance	Amount	Before	After	
Astacus:						
Normal	5	adrenaline	100γ	14.1 ± 8.3	42.0 ± 13.7 (0)	198
Normal	32	adrenaline	50	11.0 ± 4.5	29.6 ± 9.3 (0)	169
Normal	36	adrenaline	20	12.0 ± 3.6	21.9 ± 10.7 (12)	83
Normal	22	adrenaline	10	12.8 ± 3.4	20.7 ± 10.3 (9)	62
Normal	15	adrenaline	5	8.3 ± 2.8	11.9 ± 4.6 (7)	43
Normal	14	adrenaline	2	13.2 ± 4.9	18.9 ± 8.5 (6)	43
Normal	9	adrenaline	1	15.3 ± 4.2	20.2 ± 6.2 (5)	32
Normal	11	water	0.2 ml.	10.1 ± 3.3	10.1 ± 4.2 (10)	0
Normal	12	chloretone	0.1 ml.	13.1 ± 3.8	12.8 ± 4.4 (9)	0
—SG	5	adrenaline	50	6.3 ± 2.1	5.8 ± 3.8 (4)	0
—SG	6	adrenaline	100	*4.8 ± 1.4	*7.5 ± 4.1 (2)	56
—ES	8	adrenaline	100	12.2 ± 2.6	9.9 ± 2.0 (8)	0
—SG	7	adrenaline	100	14.2 ± 2.8	15.7 ± 6.4 (4)	11
Callinectes:						
Normal	6	adrenaline	150	20.5 ± 2.6	45.4 ± 4.8 (0)	121
Normal	4	adrenaline	50	8.6 ± 3.4	37.4 ± 12.1 (0)	335
Normal	7	adrenaline	10	11.2 ± 4.4	29.8 ± 11.8 (0)	105
Normal	9	adrenaline	1	13.5 ± 5.1	26.7 ± 7.0 (0)	98
Normal	5	adrenaline	0.1	18.9 ± 7.0	36.6 ± 11.5 (0)	94
Normal	12	saline	0.2 ml.	14.9 ± 4.9	29.4 ± 8.0 (2)	97
—SGN	6	adrenaline	150	15.6 ± 3.6	56.7 ± 20.9 (0)	263
—SGN	4	adrenaline	50	8.4 ± 1.3	27.6 ± 11.9 (0)	229
—SGN	4	adrenaline	10	18.6 ± 1.6	29.8 ± 7.9 (1)	60
—SGN	6	saline	0.2 ml.	10.4 ± 2.7	11.2 ± 4.5 (5)	8
—SG	5	adrenaline	150	10.8 ± 3.0	39.8 ± 10.1 (0)	269
—SG	5	adrenaline	50	18.7 ± 6.1	41.4 ± 10.3 (0)	121
—SG	5	adrenaline	10	14.3 ± 13.4	23.9 ± 8.7 (1)	67
—ES	5	adrenaline	150	14.2 ± 6.1	33.0 ± 11.4 (0)	132
—ES	4	adrenaline	50	12.6 ± 3.7	25.4 ± 12.7 (0)	102
—ES	5	adrenaline	10	15.4 ± 7.1	18.2 ± 8.6 (3)	18
—ES	4	saline	0.2 ml.	13.5 ± 4.0	11.3 ± 1.9 (4)	0

* See text.

appeared that adrenaline, or some closely related compound, might be acting at the nerve terminals in the sinus gland to induce hyperglycemia. We, therefore, examined the effect of injecting adrenaline in a series of doses ranging from 150 γ to 0.1 γ.

In the first group of normal Astacus into which 100 γ of adrenaline were injected, all 5 animals showed a marked hyperglycemia after 90 minutes; the individual glycemic values were from 1½ to 7 times the pre-injection concentration of blood-sugar, while the average increase was 198 per cent. Injection of 50 γ of adrenaline into 32 animals similarly resulted in a rise in blood-glucose in all the individuals, the post-injection concentrations ranging from 1½ to 10 times those before injection, and the average increase being 169 per cent.

With injection of lesser amounts of adrenaline, as shown in Table III, the individuals of each group became more variable in their responses. Thus, of 36 animals

injected with 20 γ of adrenaline, 7 showed slight increases ranging between 0.1 and 2.4 mg.%, 5 showed slight decreases varying between 0.9 and 4.9 mg.%, while the remaining 24 individuals demonstrated rises in blood-sugar concentration from 4.5 to 36 mg.%; the average increase over the pre-injection glycemic value was 83 per cent. Smaller quantities of adrenaline (injection of 10, 5, 2, and 1 γ) resulted in even more marked variability in response; in approximately half the number of animals of each group thus injected no appreciable increase in blood-sugar concentration occurred, while in the remainder sufficient rises in glycemic level took place to yield average increases of 62%, 43%, 43%, and 32%, respectively.

Eleven Astacus were injected with 0.2 ml. of distilled water as controls to the above experiments. Although the average for the group was the same after injection as before, the slight individual fluctuations observed in the preceding groups were also present here. Increases in glycemic concentration between 0.2 and 2.4 mg.% occurred in 4 crayfish; one animal had an increase of 3.5 mg.%; and 6 Astacus showed decreases ranging from 0.4 to 2.5 mg.%. Similar lack of hyperglycemic effect was found on injection of 0.1 ml. of saturated chloretone solution into 12 Astacus; chloretone was used as a control because the commercial adrenaline was dissolved in 0.5 per cent solution of this substance. In 8 of these injected animals decreases in blood-sugar concentration from 0.2 to 4.3 mg.% took place, while 4 crayfish showed increases ranging from 1.6 to 7.1 mg.%.

The hyperglycemic effects of adrenaline on the crayfish of this study thus seem to be unquestionable; very pronounced increases in blood-sugar concentration were obtained with doses ranging from 100 γ to 10 γ; smaller average increases were obtained with lesser doses. Injection of control substances was without appreciable effect on glycemic levels.

Several possibilities have suggested themselves as to the site of action of injected adrenaline; one, mentioned at the beginning of this section, is that adrenaline may be acting as a neurohumoral mediator, either in the central nervous system of the crustacean, or between the sinus gland nerve and the sinus gland; a second possibility is that adrenaline more or less directly effects release of glucose from an organ in which reserve carbohydrate is stored. Because of the failure of asphyxia and anesthesia to produce appreciable hyperglycemia in Astacus from which the sinus glands had been removed, as contrasted with the pronounced increases in normal animals, we decided to test the first of these possibilities by injecting adrenaline into sinus-glandless Astacus.

Five sinus-glandless crayfish were injected with 50 γ of adrenaline. In two animals increases of 0.9 and 3.7 mg.% appeared, while the remaining 3 individuals showed decreases in blood-sugar concentration of 3.2, 2.7, and 1.2 mg.%. The average blood-sugar concentration for the 5 animals, however, showed little change from that before injection, in contrast to the increase of 169 per cent obtained with identical treatment of normal crayfish. Injection of a higher dose of adrenaline, 100 γ, into one group of 6 sinus-glandless Astacus resulted in small increases of 3.9 to 6.9 mg.% in 4 animals and decreases of 2.0 and 2.7 mg.% in two. The average blood-sugar concentrations before and after injection are shown in Table III; there was an average increase of 56 per cent in glycemic concentration. While at first glance this rise of 56 per cent in animals without sinus glands might indicate the presence of a source of diabetogenic hormone other than the sinus gland, explanation of this increase may lie in other directions.

For example, this increase may have resulted from the accumulation, in the small group of experimental animals employed, of individual variations that were magnified by the low pre-injection glycemic values. We might interpolate the fact that a higher than usual set of blanks in the titration of this set of blood samples resulted in the low initial concentration of 4.8 mg.%, although the volume of ceric sulfate used in titrating the blood samples was about the same as that which usually indicated glucose concentrations between 10 and 12 mg.%. Other explanations of this same increase may, however, lie in partial regeneration (or incomplete removal) of the sinus glands, or in the action of adrenaline directly on a carbohydrate storage depot without the mediation of another endocrine secretion. To test between some of these possibilities, 8 Astacus, from each of which both eyestalks had previously been ablated, were injected with 100 γ of adrenaline. In 6 animals decreases in blood-sugar concentration (from 1.7 to 5.5 mg.%) and in 2 slight increases (1.0 and 1.4 mg.%) occurred, the average for the group being a decrease of about 19 per cent. The results do not support the possibility that adrenaline in Astacus was effecting release of glucose from a carbohydrate-storing organ. While these same results rule out the possibility of a diabetogenic hormone originating outside the eyestalk of the crayfish, they do not exclude some structure, other than the sinus gland, in the eyestalk from being a source of hyperglycemic hormone. The experiment was, therefore, repeated with a second group of 7 sinus-glandless Astacus which were injected with 100 γ of adrenaline to determine whether an increase approximating the 56 per cent rise obtained with the first group would be repeated, or whether the increase in this first group had been due to fortuitous variation. Three animals showed decreases (from 2.1 to 5.9 mg.%) while 4 showed increases (0.2, 3.4, 8.8, and 9.7 mg.%), the average for the group being a rise of 11 per cent. We are, therefore, inclined to propose, tentatively, the latter explanation of the glycemic increase in the first of these sinus-glandless groups injected with 100 γ of adrenaline. We do not believe that other factors suggested above as explanations of the increase have been decisively excluded, especially since two of the crayfish in this second group showed rather marked glycemic increases (of 8.8 and 9.7 mg.%); further study is required of this point.

Whatever may be the explanation of the slight hyperglycemia effected in sinus-glandless Astacus, comparision of the effects of adrenaline injection in normal crayfish with those of animals without sinus glands indicates quite convincingly that the adrenaline-induced hyperglycemia is mediated by way of the sinus gland.

Adrenaline injection in Callinectes was followed, in general, by hyperglycemias in normal crabs similar to those resulting in Astacus. There were, however, some interesting differences. A series of doses of adrenaline, from 150 to 0.1 γ (Table III), brought about an average increase of 100 per cent or more in glycemic concentration. A striking unanimity of response was observable in the members of each injected group, unlike the prominent variability that occurred with similar treatment in Astacus; this was especially marked with the lower dosages of adrenaline. Suspicion of this unanimity was justified when it was found that 0.2 ml. of 0.7 per cent NaCl injected into normal Callinectes was as equally effective as the lower doses of adrenaline in evoking about a 100 per cent increase in blood-sugar concentration. We believe that this is illustrative of excitement hyperglycemia due to the handling of the animals in taking blood samples and making injections; Callinectes is much more aggressive than Astacus and apparently responds more readily than does

the crayfish to these manipulations by exhibiting a marked hyperglycemia; there is no reason to suspect saline solution *per se* of any effect on blood-sugar concentration.

Confirmation of this view was obtained with the next sequence of experiments on Callinectes in which the nerves to the sinus glands had been severed bilaterally from 1 to 14 days preceding the experiment. Injection of 150 and 50 γ of adrenaline into groups of Callinectes whose sinus gland nerves had been cut 2 to 3 days and 13 to 14 days respectively brought about a net increase of more than 200 per cent in blood-sugar concentration; 10 γ of adrenaline injected into a group whose sinus gland nerves had been cut 2 to 8 days previously evoked a much smaller average increase. Injection of saline into similar animals in which the nerves had been cut 1 to 3 days yielded no significant increase in blood-sugar concentration. We, therefore, believe that the marked hyperglycemias resulting from injection of saline and of the smaller doses of adrenaline in the normal crabs were due to secretion of sinus gland hormone effected through the sinus gland nerves; the very great increases in glycemic levels obtained with the higher doses of adrenaline (150 and 50 γ) we might then attribute to the neurohumoral action of adrenaline on the denervated gland.

The inadequacy of this last interpretation was, however, demonstrated by a series of experiments whose results are in striking contrast to those obtained in practically identical tests performed with Astacus. Three groups of Callinectes whose sinus glands had been removed from 2 to 11 days preceding the experiment were injected with 150, 50, and 10 γ of adrenaline, respectively. It will be recalled from experiments described above that injection of adrenaline into sinus-glandless Astacus was attended by a modest rise in blood-sugar concentration of some individuals, no conclusive explanation for this rise being proposed at the time. Similar treatment of sinus-glandless Callinectes, however, resulted in an impressive increase in glycemic level. Hence, the explanation that adrenaline-induced hyperglycemia in animals with denervated sinus glands might be due to the action of this drug as a chemical mediator on the gland is not wholly adequate. Since this last series of experiments demonstrates a convincing hyperglycemia in animals without sinus glands, additional interpretation is not excluded. We are consequently faced with considering the presence of a mechanism in the adrenaline-induced hyperglycemia by which some structure in the eyestalk, other than the sinus gland, might be involved in the hyperglycemic response. The X-organ, a structure whose appearance of secretory activity had already impressed Hanström (1931), who originally described it in considerable detail, is one such possibility, although others may also be present.

We, therefore, explored the possibility that, in addition to the methods described above, adrenaline might be evoking hyperglycemia by acting on the X-organ or some hitherto as yet unrecognized glandular tissue in the eyestalk. Injection of the usual doses of adrenaline into eyestalkless crabs resulted in striking hyperglycemias with the two higher concentrations (150 and 50 γ) and only a very slight increase in blood-sugar after the 10 γ dose. The average percentage increases in glycemic level after the 150 and 50 γ injections were not as high as in preceding groups of experiments, but we doubt that this difference can be considered significant in view of the small number of animals in each group.

We can, therefore, conclude from the qualitative nature of the latter series of tests 1) that injection of adrenaline in Callinectes (and in Astacus) results in hyper-

glycemia; 2) that while hyperglycemia is effected through the sinus gland (anesthesia and asphyxia experiments with Astacus and Callinectes), adrenaline can also evoke hyperglycemia (convincingly in Callinectes, but much less clearly so in Astacus) in the absence of the sinus glands. Whether adrenaline may thus be acting on glandular tissue outside of the eyestalk to result in release of diabetogenic hormone, or whether adrenaline may be acting directly on tissue in which carbohydrate is stored to result in release of glucose, is as yet not known. Some of our preliminary experiments to explore these last hypotheses were too few and the results too equivocal to be discussed at this time.

Discussion

The experiments described above demonstrate the necessity of the sinus gland for asphyxia- and anesthesia-induced hyperglycemia to occur. Failure of hyperglycemia to appear after asphyxia in animals whose sinus gland nerves had been cut indicates that the response is part of a reflex mechanism. The details of the mechanism of chloroform-induced hyperglycemia were not explored beyond indicating its dependence on the presence of the sinus gland; study of the sequence of effect of anesthesia on the centers of the crustacean nervous system could not be critically assessed at this time and were beyond the scope of this investigation. The appearance of Needham's (1950) paper supplies some of this information. This author found that the neurogenic heart of decapod crustaceans is arrested by ether and chloroform anesthesia earlier than are the somatic movements; complete quiescence of the heart for periods up to an hour was not attended by fatal consequences, recovery, in the case of Carcinus, taking 1 to 2 hours. The circulatory stasis accompanying this cardiac arrest must build up a high oxygen debt. The effect of chloroform anesthesia on the sinus gland may thus be mediated by the same centers of the nervous system that are involved in the hyperglycemia resulting from asphyxia.

Conclusive evidence for the activity of chemical mediators of nervous transmission in crustacea has not been abundant. Some studies (for review see Bacq, 1947) reporting the presence of such mediators have been acute experiments involving perfusion of isolated organs, or the injection of acetylcholine or adrenaline into the organism, as was done with the latter substance in this study. Experimental investigations of this nature are actually little more than reports of the pharmacological effect of the injected substances; they do not prove the role of such substances as chemical mediators in the physiology of the organism, but indicate only the possibility of such a role. Prosser (1942) has presented criteria to be observed in considering the physiological role of acetlycholine. Comparable standards could be established to define the relation between adrenaline and the presence of adrenergic nerves. Bacq has in fact reviewed critically a number of studies concerned with the effect of acetylcholine and sympathomimetic amines among invertebrates.

Bacq's view that invertebrate tissues show a non-specific and often low sensitivity to adrenaline and the sympathomimetic amines might be modified in the light of more recent information. His view was based in part on the contradictions in the literature, already summarized in the introduction above, on the hyperglycemic effect of adrenaline in crustacea. When Kalmus and Waldes reported in 1936 that the hyperglycemic response in crustaceans was non-specific, being just as

readily evoked by injection of physiologically irrational substances as by adrenaline, the importance of the sinus gland as an endocrine organ and its being a source of diabetogenic hormone was not yet known. The demonstration in this study of the role of the sinus gland in excitement hyperglycemia may explain the results of Kalmus and Waldes; thus, their questioning of the specificity of the adrenaline-induced hyperglycemia would become instead a question as to whether these non-specific substances were effecting hyperglycemia by acting through the nervous system to bring about release of diabetogenic hormone from the sinus glands.

Bacq's point concerning the low sensitivity of invertebrate tissues to adrenaline might not seem applicable to the adrenaline-induced hyperglycemia described here. The lower threshold of hyperglycemic doses of adrenaline for the Astacus of this study was between 1 and 5 γ. If, for conveniently simplifying the calculations, we consider the crayfish used to have weighed 100 grams, the blood-volume of such animals would be about 25 ml., according to the determinations of Prosser and Weinstein (1950) on Cambarus. If the injected adrenaline is uniformly mixed and persists *in toto* in the blood without being partially eliminated or inactivated, the concentration of adrenaline in the vascular system of the animal that brings about an appreciable increase in the glycemic level is then about 10^{-7}. Sensitivity to such concentrations of adrenaline is not far removed from that shown by some isolated organs or tissues used in the assay of chemical mediators of nervous transmission.

It is obvious that isolated observations, such as those above, do not constitute adequate evidence for the existence of adrenergic nerves in crustacea, but they do remove some of the criticisms that had been levelled against such observations in the past. Criteria for conclusive proof of the existence of adrenergic nerves can be established with which to compare results from future experimental procedures aiming toward this end.

Summary

1. Asphyxia of normal Astacus and Callinectes results in marked increase in concentration of blood-sugar; such increase is mediated by sinus glands located in the eyestalks, for after removal of these glands hyperglycemia does not occur after asphyxia. Hyperglycemic response to asphyxia is part of a reflex mechanism; it does not occur following denervation of the sinus glands.

2. Chloroform anesthesia in Astacus similarly evokes rises in glycemic level which fail to occur after sinus gland removal. Since anesthesia brings about cardiac arrest, the resultant anoxia may be the basic explanation of the anesthesia-induced hyperglycemia.

3. Adrenaline, in doses from 1 to 100 γ, causes hyperglycemia in normal Astacus, but injection into sinus-glandless crayfish shows either no rise in the concentration of blood-sugar, or a very slight one. Similar injections into Callinectes result in marked hyperglycemia in normal individuals, in animals with denervated sinus glands, in sinus-glandless and also in eyestalkless individuals.

4. The results of this study indicate the presence of an excitement hyperglycemia in these crustaceans, ensuing from release of diabetogenic hormone from the sinus glands. The effects of adrenaline in Callinectes may be due to its action on glandular tissue outside of the eyestalk, bringing about secretion of diabetogenic hormone

(in addition to its effect on the sinus glands), or to its action on carbohydrate-storing tissue resulting in release of glucose.

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GLYCOGEN DEPLETION DURING STARVATION IN THE NEMERTEAN, MICRURA LEIDYI (VERRILL), AND ITS ECOLOGICAL SIGNIFICANCE [1]

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Nemerteans, free-living organisms sometimes popularly known as ribbon-worms or proboscis-worms, are able to survive long periods of complete starvation under aerobic conditions. Coe (1943) reported the survival of some species of Lineus, Prostoma and Procephalothrix for more than a year. During this time the body was greatly reduced in size by the absorption of tissues from the gonads, digestive system and proboscis. Incidental observations were made by Jacubowa and Malm (1931) on worms identified as "Nemertinae" under anaerobic conditions. They noted survival for more than five days in a fouled, oxygen-poor aquarium. In his review of anaerobiosis, von Brand (1946) indicated that this may not have been an oxygen-free environment. He further emphasized the paucity of knowledge of the metabolism of various groups of invertebrates. This lack of information is especially obvious in the phylum Rhynchocoela (Nemertea) where no studies besides those reviewed above have been conducted.

While comparing the glycogen metabolism of various species of parasitic helminths, sufficient numbers of *Micrura leidyi* (Verrill), 1892, were available to study the glycogen metabolism in worms: (1) analyzed directly after collection from the ocean, (2) starved under aerobic conditions for 16 to 35 days, and (3) starved under completely anaerobic conditions for 24 to 72 hours. In addition, observations on other effects of longer anaerobic starvation were made.

Materials and Methods

Specimens of *Micrura leidyi* were obtained during July and August by digging in protected sandy bottoms near the low tide mark in the Woods Hole area. The substrate varied from almost pure sand to sandy mud. Other prominent invertebrate organisms in the same ecological association were the holothurian, *Leptosynapta inhaerens,* the molluscans, *Mya arenaria* and *Venus mercenaria,* and several species of polychaete annelids. The nemerteans were transported to the laboratory in individual bottles and experimental procedures initiated as soon as possible. Unfortunately *M. leidyi,* when handled, secretes large quantities of mucus and has a tendency to fragment. As reported by Coe (1943), rapid regeneration of a new caudal cirrus occurs if the anterior end is present. Fragments from the posterior end do not regenerate and soon begin to decay. These disadvantages were counterbalanced by availability and by the convenient size of the organism for weighing and making glycogen determinations on single specimens. Fragmentation and mucus

[1] This study was partially supported by a grant from the American Association for the Advancement of Science awarded by the Illinois State Academy of Science.

secretion usually ceased within 24 hours after the worm was placed in a container although some specimens continued to fragment and had to be discarded.

For aerobic starvation, worms were placed in filtered sea water in large, covered, crystallizing dishes and stored in a dark room at 18° C. In anaerobic starvation worms were placed in individual bottles of sea water which had been boiled and cooled to 23° C. (temperature of sea water circulated around the bottles). These bottles were stoppered and sealed except for glass tubing connections through which hydrogen or nitrogen was bubbled using the methods of Michaelis and Flexner (1928) to eliminate all traces of oxygen. The assistance of Dr. E. S. G. Barron in making available the apparatus to provide oxygen-free gases is gratefully acknowledged.

Glycogen analyses were made using the combination of methods reviewed by Reid (1942). However, glucose determinations were made with the aid of a photoelectric colorimeter using the standard method of Folin-Malmros.

Experimental

Aerobic starvation. As living worms became available they were divided into three groups. The experimental group was weighed and starved in filtered sea water as long as time at Woods Hole would permit before making a glycogen analysis. The average glycogen level in seven worms was 1.83 per cent after 16 to 35 days of starvation (see Table I). One control group was analysed for glycogen as soon as the worms were dug to determine the food reserve maintained by this animal in its natural environment. Eight worms averaged 1.57 per cent glycogen.

In the original design of the experiment a second control series was set up to study possible deleterious effects of confinement in a smooth, glass vessel. After weighing, the animals were placed in sea water in some of the sand in which they normally burrow. However, three out of four died in a few days. The one survivor, when reweighed after 35 days in the sand, showed a weight loss of 33 per cent as compared to an average loss of 10 per cent in the experimentally starved group. These results are probably caused by loss of a fragment in the sand. Since the experimental group after 35 days in a glass container appeared normal and moved with the usual gliding movement of this species, the second control series was discontinued.

As indicated above, starved worms contained slightly more glycogen than the controls, but this increase is not statistically significant. The fact that there is no decrease during starvation indicates that this organism does not depend upon polysaccharide metabolism during aerobic starvation.

Worms were measured and also weighed to determine decreases in size during the experimental, aerobic starvation. Because of the extensibility of the specimens, length and width measurements proved less reliable than weights obtained on an analytical balance. The loss in weight varied from 3.3 to 17.2 per cent, with an average of 10 per cent for the seven worms. Controls averaged 0.6383 grams total weight at the time of glycogen analysis while experimentals averaged 0.5949 grams.

Anaerobic starvation. During the entire anaerobic period some activity was apparent. The slow gliding movement, probably caused by cilia, could best be detected at the anterior end. Swimming movements are not characteristic of this

species and were never observed. One specimen survived seven days of complete anaerobiosis but was dead on the eighth day. Anaerobic conditions frequently induced an increase in fragmentation. Since these pieces decayed and could not be removed without disturbing the condition of anaerobiosis, toxic products may have shortened the survival time. Four specimens were analyzed at the end of 24 hours of anaerobiosis and found to contain 1.49 per cent glycogen. Since this figure was

TABLE I

Glycogen level in starved and unstarved Micrura leidyi

Treatment	Duration of treatment	Weight loss during treatment	Percentage of glycogen (wet weight)	Average glycogen for group
Unstarved controls analyzed immediately after removal from the ocean (eight worms)	—	—	1.70	
	—	—	1.81	
	—	—	0.88	
	—	—	1.55	
	—	—	1.94	
	—	—	1.62	
	—	—	1.91	
	—	—	1.18	1.57 ± 0.13 (S.E.)
Control kept in sand (one worm)	35 days	33.0%	2.42	2.42
Aerobic starvation (seven worms)	35 days	17.2%	1.53	
	35 days	10.1	1.54	
	23 days	15.2	1.96	
	23 days	4.0	1.50	
	23 days	4.1	2.46	
	23 days	3.3	1.50	
	16 days	15.0	2.32	
				1.83 ± 0.16
Anaerobic (four worms) starvation	24 hours	not determined	1.10	
	24 hours		1.97	
	24 hours		1.35	
	24 hours		1.53	
				1.49 ± 0.18
(four worms)	72 hours	not determined	0.17	
	72 hours		0.38	
	72 hours		0.44	
	72 hours		0.16	
				0.29 ± 0.07

lower, but not low enough to show a statistically significant decrease over the controls, a second series of four worms was starved 72 hours and analyzed. The decrease in glycogen level to 0.29 per cent is statistically significant and indicated an essential role of glycogen in anaerobic starvation of the species. Individual weights of worms averaged 0.7964 grams in the 24 hour group but those starved 72 hours averaged only 0.1488 grams. This average, much lower than the controls, was due to the elimination of fragments before glycogen determinations. Weight loss

during starvation was not determined because handling prior to initial weighing increased the rate of fragmentation, as mentioned above, and made the worms less suited for anaerobic treatment.

Discussion

The property of enduring long periods of starvation by subsisting on food reserves or tissues is known in a number of invertebrate organisms. Planarians have been the subject of many investigations of this sort. Using histochemical methods Hyman, Willier and Rifenburgh (1924) found no glycogen in *Planaria dorotocephala*. Von Brand (1936) found glycogen in *Planaria torva* which varied from 3 per cent of the wet weight in the Fall to 0.5 per cent during May. This glycogen reserve is reduced to a trace after one week of aerobic starvation. These two studies indicate a less essential role of glycogen and consequently poorer adaptation to anaerobiosis in the economy of this organism than that operative in *M. leidyi*.

In various parasitic helminths the role of glycogen has been under intensive investigation since it is known that large quantities of this polysaccharide are stored by many species. The glycogen reserve, which is much higher in most parasitic forms than in *M. leidyi*, has been interpreted as an adaptation which permits survival under conditions of anaerobiosis in the gut of the host. Hopkins (1950) found as much as 16 per cent of the wet weight of the cestode *Schistocephalus solidus* was glycogen. This reserve is often utilized rapidly under starvation conditions. According to Reid (1942), the cestode *Raillietina cesticillus* used 95 per cent of its stored glycogen when the host was starved 24 hours. Comparison between parasites and free-living forms can be made only in cases where satisfactory *in vitro* techniques of experimentation make possible the control of the oxygen tension during starvation. The larval nematodes, *Eustronglylides ignotus*, utilized glycogen aerobically and more rapidly anaerobically according to von Brand and Simpson (1945).

From the scanty information available, *Micrura leidyi*, with its ability to reserve glycogen for periods of anaerobiosis, may resemble leeches which are reported to have a predominately protein metabolism during starvation. However, as pointed out by von Brand (1946), almost nothing is known of the carbohydrate metabolism in this group. Until this field and the protein metabolism of nemerteans has been investigated no extensive comparison is possible.

Micrura leidyi appears to differ from planarians and the parasitic groups studied by maintaining, under conditions of aerobic starvation, a glycogen reserve which is available to assist in survival under anaerobic conditions. Nemerteans may frequently encounter oxygen-poor conditions for short periods of time in their natural environment. As indicated by von Brand there may be periods of anaerobiosis during low tide for many marine forms. Furthermore, this species is found in burrows deep in sandy-mud where it may be subjected to oxygen-poor conditions. The ability to survive both aerobically and anaerobically without food for relatively long periods is an adaptive mechanism which has probably contributed to the survival of this species.

Summary

1. *Micrura leidyi* maintains a glycogen store of 1.83 per cent of the wet weight of the worm after 16 to 35 days of aerobic starvation. Specimens removed directly

from the ocean had 1.57 per cent glycogen. The ability of an organism to reserve glycogen for use under anaerobic conditions is a possible adaptive mechanism which has not previously been reported.

2. The glycogen level decreased from 1.57 per cent to 1.49 per cent after 24 hours and was further reduced to 0.29 per cent after 72 hours of anaerobiosis.

3. *M. leidyi* is relatively resistant to conditions of complete anaerobiosis. The maximum survival time in the present experiments was seven days.

4. Seven specimens starved aerobically for 16 to 35 days lost an average of 10 per cent of their original body weight.

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NOTES ON OVULATION, OVA, AND EARLY DEVELOPMENT IN THE SMOOTH DOGFISH, MUSTELUS CANIS[1]

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Although there have been a number of studies on development and related problems in the ovo-viviparous *Squalus acanthias* (Scammon, 1911; Ford, 1921; TeWinkel, 1943; Hisaw and Albert, 1947) and in oviparous rays, skates, dogfishes, and sharks (Clark, 1922; Vandebroek, 1936; Metten, 1939; Smith, 1942) there has been almost no recent embryological work on viviparous elasmobranchs other than that treating of utero-gestation (ten Cate-Hoedemaker, 1933; Ranzi, 1932, 1934). This lack of information is readily explained by the difficulty of collecting adequate material. During several summer periods at the Marine Biological Laboratory, Woods Hole, Massachusetts, while collecting embryos of the American smooth dogfish, *Mustelus canis*, for another purpose, certain observations were made on the breeding season, ova, embryonic orientation, and rate of development in this species. These observations serve as the basis for the present report.[2]

Mustelus canis (Mitchell) (Hubbs, 1938) is the only species of Mustelus commonly found along the Atlantic coast of North America. Hubbs considers it to be closely related to the European smooth hound, *M. laevis*. Both species are members of the family *Galeorhinidae* under the order *Galea* (White, 1937). *Mustelus canis* arrives in the vicinity of Woods Hole during May and June and may be collected through August and possibly September, but presumably seeks warmer waters in late autumn. Bigelow and Welch (1924) state that this species is rarely seen north of Cape Cod and that its range extends from Cape Cod to Cuba.

MATERIAL AND METHODS

Mature female specimens of *Mustelus canis*, usually 2½ to 4 feet in length, weighing 5 to 20 pounds, were collected chiefly by fishing from the docks in Woods Hole or from a small boat, provided by Mr. James McInnis of the Supply Department, during the summers of 1945, 1946, 1948, and 1949. Many fishing enthusiasts cooperated in this enterprise but special acknowledgment should be made to Dr. R. T. Kempton who contributed a large number of specimens used in his own experimental work on elasmobranch excretion. Specimens were placed in live cars or in large outdoor aquaria supplied with running sea water. Dr. Kempton's specimens were kept for several days, during which time eggs were occasionally crushed and often extruded. For optimum material fish were opened within twenty-four hours

[1] Contribution from the Department of Zoology, Smith College, No. 227.

[2] The genus *Mustelus* has attracted attention embryologically because of the presence of a yolk-sac placenta during much of its extended gestation period (beginning when embryos are 10–16 cm. in length, Ranzi, 1934). In specimens of *M. canis*, collected between June and late August, the placenta had not yet developed.

TABLE I—*Summary of records of embryonic stages in 60 specimens of Mustelus canis*

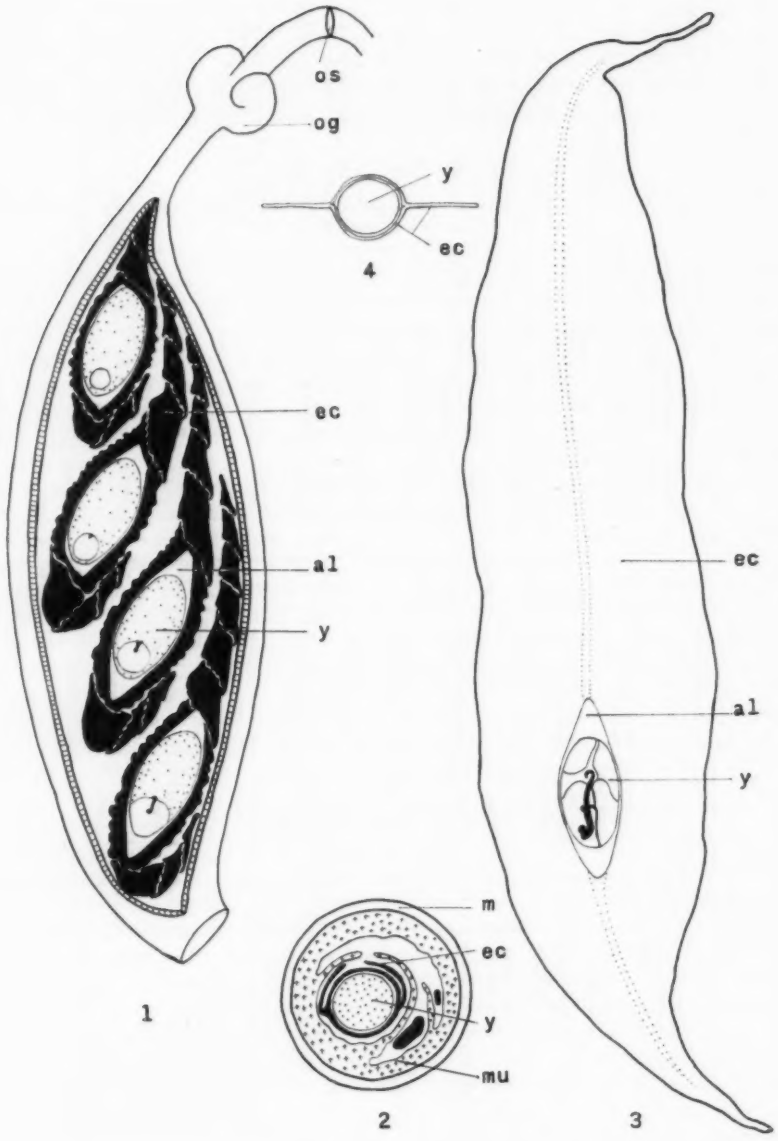
June				July				July				Aug.			
Date	No. ♀s	No. ut. ova	Notes on embryos	Date	No. ♀s	No. ut. ova	Notes on embryonic stages	Date	No. ♀s	No. ut. ova	Notes on embryonic stages	Date	No. ♀s	No. ut. ova	Notes on embryonic stages
8/45	8	1 in one ♀; none in 7 ♀s	all ♀s pp.; ovaries with 12 to 24 large ova	3/45	1	6 each uterus	st: bl. to f.n.f.	14/49	1	7 each uterus	st: bl. to 5 or 6 mm. embryos	1/46	1	5 each uterus	st: 18 to 28 mm. embryos
				3/49	1	7 rt; 6 lft. +1 empty case ant.	ov. comp.; st: cl. to m.n.f.	16/46	1	3 each uterus	st: 10, 13, 18 mm. embryos	4/46	1	5 one ut.; 5 empty cases other ut.	st: 15 to 28 mm. embryos
16/45	1	none	pp., large ova in ovary					16/49	1	9 rt. (1 d.y.) 8 lft.	5 ova in ovary; st: bl. to 7 or 8 mm. embryos				
25/46	1	6 each uterus	several ova in ovary; st: t.g.d. to bl.	7/45	1	7 one uterus; 5 other	st: bl. to 5 mm. embryo	17/46	1	5 each uterus	st: 7 to 15 mm. embryos		1	5 each uterus	st: 15 to 25 mm. embryos
25/49	1	4 each uterus	4 ova in ovary; st: cl. to bl.		1	7 one uterus; 8 other	st: cl. to m.n.f.	18/46	4	approx. 5 each uterus	st: 1 ♀, bl. to 8 mm. embryos; 3 ♀s, 6 to 18 mm. embryos	4/48	1	8 each uterus	ov. comp.; st: 12 to 27 mm. embryos
27/46	1	1 each uterus	several ova in ovary; st: t.g.d.	7/46	1	7 each ut. (d.y. each ut.)	ov. comp.; st: bl. to 8 mm. embryos					9/46	1	no data	st: 28 to 40 mm. embryos
28/45	1	5 each uterus	st: cl. to bl.	7/49	1	5 one ut.; 4 + case ant.	st: f.n.f. to 10 mm. embryos	19/48	1	6 each uterus	st: condition too poor to tell	13/48	1	4 each uterus	ov. comp.; st: 57 to 82 mm. embryos†
					1	6 each uterus	st: e.n.f. to 8 mm. embryos		1	8 one uterus; 9 other	st: cl. to 3 mm. embryo	14/48	1	8 one ut.; 5 other (ex.)	st: 23 to 34 mm. embryos + 1 crushed
				9/46	1	5 one ut.; 6 (1 d.y.) other	ov. comp.; st: 3.5 to 10 mm. embryos	20/48	1	7 one uterus; 6 other	st: g. to 8 mm. embryos	16/48	1	6 each uterus	st: 35 to 47 mm. embryos
				9/49	1	6 each uterus	st: f.n.f. to 11 or 12 mm. embryos	21/48	1	4 each uterus	st: f.n.f. to 8 mm. embryos	30/46	2*	total of 6 in 1 ♀; 11 in other	st: approx. 45 to 70 mm.; yolk-sacs nearly empty
				12/48	1	4 one; 2 other (ex.)	ov. comp.; st: bl. to e.n.f.	23/48	1	8 each uterus	st: g. to 10 mm. embryos				
				13/48	1	7 one; 5 other (ex.)	ov. comp.; st: cl. to g.; post. 3 ova crushed	24/48	1	7 each uterus	st: 4 to 20 mm. embryos	31/46	1*	total of 9	st: 50 to 70 mm. embryos; yolk-sacs nearly empty
				13/49	1	7 each uterus	st: e.n.f. to 10 mm. embryos	26/48	1	6 each uterus	st: 6 to 20 mm. embryos				
				14/45	6	No data	st: 2 groups: 1) bl. to n.f. 2) 4 to 12 mm. embryos	29/48	1	4 one uterus; 5 other	st: 12 to 22 mm. embryos				
								31/48	1	4 in all (ex.)	st: 13 to 18 mm. embryos				
									1	2 one uterus (ex.)	st: 15 and 18 mm. embryos				

Key:

bl.	blastodisc
cl.	cleavage
d.y.	"double-yolked" egg
e.n.f.	early neural folds
ex.	extrusion probable
f.n.f.	fusing neural folds
g.	gastrulation stages
m.n.f.	mid-neural folds
ov. comp.	ovulation of large ova completed
pp.	post-partum
st.	embryonic stages
t.g.d.	terminal germinal disc during or before cleavage
ut.	uterus

† Embryos unusually large for date.

* Specimens contributed by Dr. Donald Zinn.



Figures 1–4

of collecting; uteri and oviducts were tied off and removed to bowls of sea water for detailed observation. Eggs in their cases were transferred from the opened uterus to finger bowls of sea water set in running sea water (22 to 23.5° C.).

Several methods were employed to keep embryos alive, including: 1) a slow drip of sea water into the dishes; 2) covering the dishes and changing or aerating the water several times daily; 3) Elasmobranch Ringer's solution, with or without urea, changed or aerated; 4) pasteurized sea water aerated several times daily; 5) eggs in cases were placed on wet towelling, or agar, or wet cotton in covered dishes and rinsed in sea water periodically.

Cleavage and blastula stages never appeared to develop further; older stages often lived 24 hours and many young motile embryos survived 40 to 50 hours, while a few 20 mm. embryos remained active for 3½ to 4 days. In the salt solutions, disintegration was first apparent in the yolk-sac which puckered peripherally and gradually shriveled while albumen became opaque or milky. The embryo often remained active for 10 to 24 hours after yolk-sac damage had begun. Moist chambers were somewhat better than immersion methods, particularly in gastrulation and neural fold stages which lived up to 48 hours; shrivelling of the yolk-sac and putrefaction of the yolk resulted in death.

Notes on Female Reproductive Organs

As in many elasmobranchs (Daniel, 1934), only the right ovary of *M. canis* functions in the production of ova while the vestigial left ovary is represented by a strip of tissue that may be glandular. In early June the right ovary contains approximately 12 to 24 large ova ready for ovulation (Table I).

The two oviducts unite in a common ostium situated ventrally at the anterior border of the liver. A thick-walled oviducal gland is present near the anterior end of each oviduct and fertilization presumably occurs as the egg passes through this gland which secretes albumen and the egg-case (Metten, 1939). Approximately ⅕ths of each oviduct is differentiated as a uterus opening posteriorly into the cloaca. The most noteworthy feature of the uterus in early gestation is its mucosa, a smooth, loose, highly edematous layer which can be easily stripped from the muscle coats. Thin folds of the loose mucosa extend into the lumen of the uterus and wrap around the eggs in their cases.

Eggs and Egg-Cases

In the ovary, the ovum of *M. canis* is spherical but during its passage through the oviduct and oviducal gland, it is compressed into an ellipsoid shape. Such ellipsoid ova are 30 to 38 mm. in their longest diameter and 17 to 20 mm. wide and have a volume of 2.3 to 2.8 cc. and a weight of approximately 2.9 grams in the fresh condi-

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Figure 1. Semi-diagrammatic drawing in ventral view of the right oviduct and uterus of *Mustelus canis* to show orientation of egg-cases and embryos. Approximately × one-half but embryos are proportionately enlarged for the sake of clearness.

Figure 2. Diagrammatic transverse section of uterus (Fig. 1) to show folds of uterine mucosa inwrapping egg-case.

Figure 3. One egg-case of *M. canis* spread out to illustrate its dimensions and the position of the yolk. (× one-half.)

Figure 4. Transverse section of egg-case (Fig. 3) cut through the ovum. al, albumen; ec, egg-case; m, muscle; mu, mucosa; og, oviducal gland; os, ostium; y, yolk.

tion. The ovum is bright yellow and by the time it reaches the uterus it has been enclosed in 1 to 2 cc. of albumen and a brownish-yellow, transparent, flexible egg-case, the free ends of which are spirally twisted (Fig. 1). This twisted condition suggests that the egg was rotated during its passage through the oviduct and possibly the uterus as well. When the egg is placed in a bowl of sea water, the spiral ends of the case tend to uncoil (Fig. 5). The case is flat except where the egg is contained (Fig. 4); it tapers at either end and, completely spread out, measures 6 to 8 cm. in width and 35 to 48 cm. in length (Fig. 3).

FIGURE 5. Photograph of the living ovum and egg-case of *Mustelus canis*, actual size. From a Kodachrome taken by George G. Lower. (Used by permission of Mr. Lower.)

During the first few weeks of gestation, the egg is confined to a region approximately 12 cm. from the posterior end of the case making possible an accurate determination of the orientation of an egg-case in utero. After the albumen has been absorbed, however, and the embryo is 50 to 70 mm. long (7 to 9 weeks old) the adjacent walls of the case split apart and the embryo with its yolk-sac can be made to pass freely from one end to the other showing the extent of the egg-case cavity. Whether the wall of the case remains intact during the entire gestation period can be determined only from late winter or early spring specimens, not available for the

present study, but its dimensions imply such a possibility. Each egg-case is almost completely wrapped by thin folds of uterine mucosa which thus form partitions between adjacent ova as shown diagrammatically in Figure 2.

Orientation of the Blastoderm

In the earliest stages of uterine ova examined, the germinal disc is usually found at the extreme posterior tip of the egg suggesting that the egg passes into and through the oviduct with the animal pole in the advance position (Fig. 6, A). During late cleavage, however, shifting of the yolk brings the blastoderm to a less restricted region (Fig. 7, Ser. A). The characteristic orientation of the blastoderm,

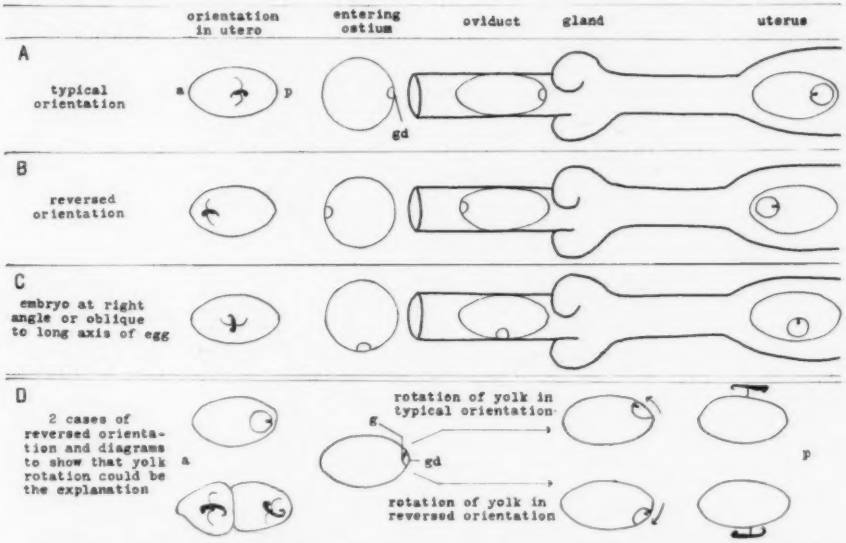
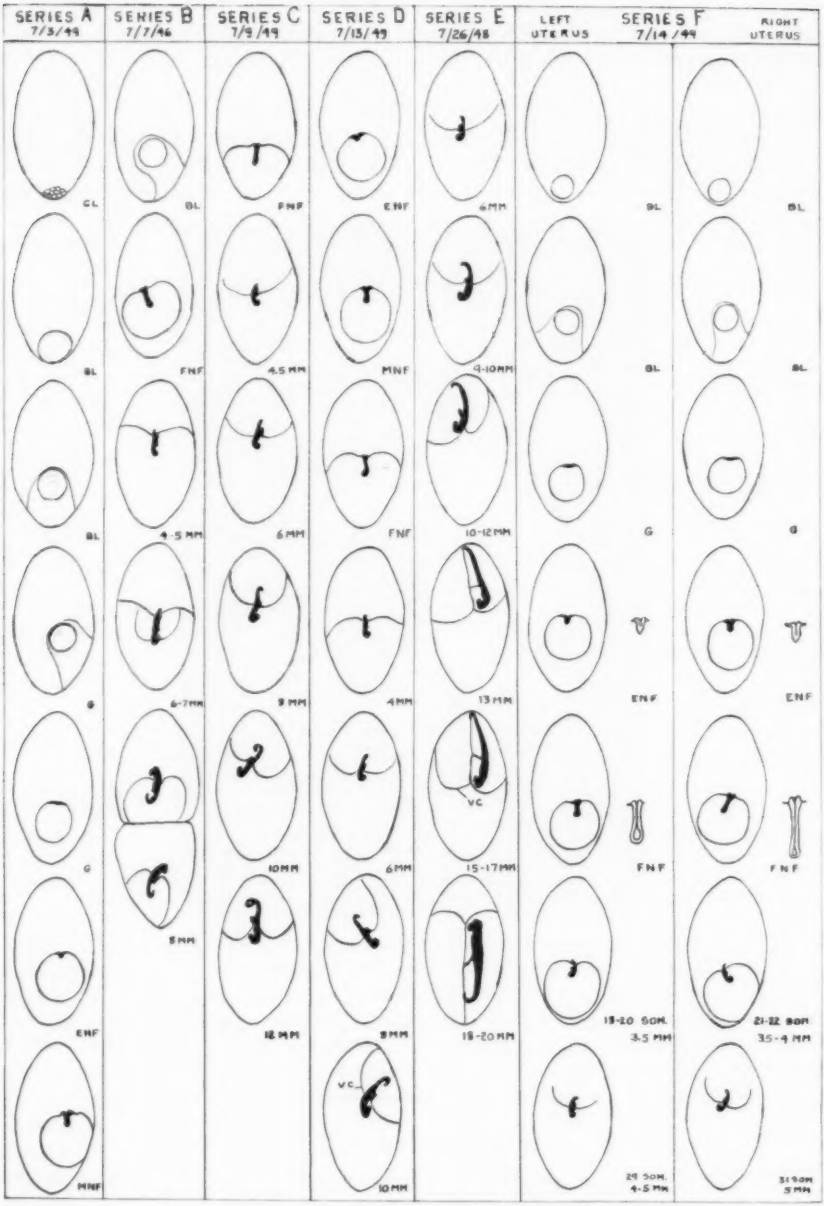


Figure 6. Diagrams A–C illustrate probable orientation of ovum on entering and passing through oviduct as determined from orientation of embryo observed in utero. Diagram D is an alternate explanation of reversed orientation. All ova are oriented similarly. a, anterior; g, gastrulation; gd, germinal disc; p, posterior.

dorsal lip, and median axis of the embryo are illustrated in Figures 1 and 7. Typically the cephalic end of the embryo lies nearest the posterior end of the egg-case. Occasional exceptions to the typical orientation occur in which the embryo faces anteriorly or in which it lies at a right angle or oblique to the long axis of the egg. Possible explanations of such exceptions are offered in Figure 6 (B, C, D). Only early stages when the blastoderm is clearly defined and before the yolk-sac has spread extensively are reliable for determination of embryonic orientation.

Ovulation Period and Rate

Mustelus canis has a gestation period of about 10 months' duration. Pups, about 1 foot (30 cm.) in length, are born according to reports in May and pos-


SERIES A
7/3/49
SERIES B
7/7/46
SERIES C
7/9/49
SERIES D
7/13/49
SERIES E
7/26/48
LEFT UTERUS
SERIES F
7/14/49
RIGHT UTERUS
CL
BL
FNF
ENF
6MM
BL
BL
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4.5 MM
MNF
9-10MM
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VC
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FNF
FNF
ENF
12 MM
8MM
18-20 MM
19-20 SOM.
3.5 MM
21-22 SOM
3.5-4 MM
MNF
VC
10MM
29 SOM.
4-5 MM
31 SOM
5 MM

FIGURE 7

sibly during late April and early June. No information is available regarding the lapse of time between birth of a "litter" and the succeeding period of ovulation but, in specimens examined in the present study, only one case of a single ovulation was observed prior to June 17 while after June 25, all mature females were pregnant. Thus an interval of 4 to 6 weeks between birth and the beginning of the next ovulation period seems a reasonable estimate.

Table I summarizes the findings in 60 specimens collected during several summers between June 8 and August 31. The number is small and the gaps which exist are recognized but, in view of the difficulty of securing material, could not be avoided. Observations, on the other hand, are consistent and are offered as representative of conditions in this species.

Data indicate that ovulation in *M. canis* usually begins in the last week of June. Specimens examined during this period contain recently ovulated eggs in cleavage and early blastoderm stages and large ova still to be extruded from the ovary. During the first two weeks of July, occasional cleavage stages are present while the oldest embryos range from neural fold stages to 10 or 12 mm. embryos. The ovary sometimes contains large ova but more frequently is totally devoid of them. After July 20, most embryos are beyond neural fold stages and in some cases measure 18 to 20 mm. in length; only one specimen containing early blastoderms was found. In August, the youngest embryos are 12 to 18 mm. and the oldest, 70 to 82 mm. in length (Table I).

From records on the condition of the ovary and embryonic stages present in utero, it may be inferred that mature females generally ovulate between late June and mid-July. Further discussion of this point will be found at the end of the section on rate of ovulation.

Calculations on the rate of ovulation are based: 1) on the series of embryonic stages in utero and 2) on the rate of development of living embryos.

1) Series of stages in utero. Figure 1 demonstrates the condition observed in *M. canis*, and said to be found in certain other viviparous elasmobranchs, that the eggs in a given uterus differ markedly in developmental stage. In *M. canis* the egg nearest the oviducal gland is always the earliest stage present and posterior to it are eggs in progressively more advanced stages arranged in sequence to the cloacal end. Figure 7 shows several examples of such series recorded in the present survey.

A second point of interest is the fact that in a given female, there is close similarity between the series of stages in the right and left uteri (Fig. 7, Series F). This condition is so characteristic that it warrants the assumption that as a rule two eggs are ovulated simultaneously or in rapid succession, one usually passing into

FIGURE 7. Each vertical column, A–E, represents the series of embryonic stages contained in one uterus of a specimen of *M. canis* on the date recorded. Ova are arranged in the sequence and orientation observed in utero but embryos are proportionately enlarged. The most posterior figure in Series B shows a "double-yolked" egg. Circular blastoderms in several figures are surrounded by an area lighter in color than the rest of the yolk. The periphery of the yolk-sac is not shown in older stages as it spreads rapidly and is increasingly difficult to distinguish. Early vitelline blood vessels are indicated. The two vertical columns of Series F represent embryonic stages from the left and right uteri of a single specimen to show the similarity in stage of corresponding embryos, indicating that ova are ovulated in pairs. bl. blastodisc; cl, cleavage; enf, early neural folds; fnf, fusing neural folds; g, gastrulation; mnf, mid-neural folds; vc, vitelline circulation.

each oviduct. Metten (1939) reports that oviparous dogfish commonly lay eggs in pairs and that two ova leave the ovary together. Ovulation of two eggs in *M. canis* is undoubtedly followed by a period of rest after which two more eggs are extruded and so on until all large ova have been discharged. In the oviparous *Raia brachyura,* Clark (1922)[3] noted that one egg capsule emerged from the cloaca to be followed immediately by another after which a lapse of about 24 hours intervened before the next spawning took place. Clark remarks that this is in keeping with the maturation of a single egg from each ovary. In *M. canis* both eggs are ovulated from the right ovary since the left is non-functional.

Rarely, in *M. canis,* are two eggs contained in a common egg-case and invariably such "double-yolked" eggs are in similar stages of development, clearly suggesting that they were ovulated simultaneously and possibly were held together by common tissue so that they passed into one oviduct. In one specimen (7/7/46) each uterus contained a "double-yolked" egg in the most posterior position and 4 eggs in the remainder of the series (Fig. 7, Ser. B). All four embryos of the double eggs were approximately 8 mm. in length suggesting that in this instance four eggs must have been discharged at about the same time, two entering each oviduct.

No data were collected on the migration of ova from the ovary to the ostium as none was seen during this period. Metten's (1939) interesting observations show that in *Scylliorhinus* this process is accomplished through the action of peritoneal cilia and that it is probably completed in a "matter of hours."[4]

2) *Rate of development of living embryos.* Study of the rate of development in *M. canis* was limited by the scarcity of material, its use for other purposes, and especially by the difficulty in keeping embryos alive (see Methods). Germinal disc stages and early blastoderms never appeared to develop further under laboratory conditions. Stages in which the blastoderm was thickened at its posterior margin (the beginning of the dorsal lip in gastrulation) advanced to early neural folds in approximately 35 hours at 22 to 23° C. Very early neural fold stages elongated but folds had not begun to fuse in 35 hours. Stages with well developd folds elongated and folds were fusing except at the anterior end in 35 hours. A stage with folds fused except anteriorly had formed a moving embryo with 19 to 20 pairs of somites in 12 hours (Fig. 8). In summary, an embryo would advance from the period of gastrulation to a completely fused tubular embryo in about five days.

From observations made to date, therefore, it is estimated that adjacent embryos in a given uterus in *M. canis* are at stages usually differing from each other by approximately 36 hours of developmental time. Because of the opacity of the yolk, embryos beyond late neural fold stages could not be compared in the living condition except in length. In a typical series (7/16/49) embryos measured: 3.6, 5, 6, and 8 mm. If embryos are removed from the yolk and the somites counted, the following record is typical of a series: 16–17, 29, 38, 50, 65–68, 83 somites.

Above data on the rate of development in *M. canis* are supported by the work of Vandebroek (1936) who noted in the oviparous *Scyllium canicula* (at 16° C.) that three days elapsed between the earliest sign of neural folds and the fused neural tube.

[3] P. 583.
[4] P. 230.

Incidental observation by the writer in 1946 showed that two eggs of the skate, *Raia erinacea,* spawned on July 13 or 14 in the aquarium of Dr. F. A. Hartmann and kindly contributed by him, were in open neural fold stages on July 21. Neural folds fused and embryos elongated from 3 to 5 mm. at the rate of about 0.5 mm. per day over a period of 3½ days and moved laterally by rhythmic contraction of somites. Unfortunately accidental injury to the egg-cases resulted in the death of the embryos.[5]

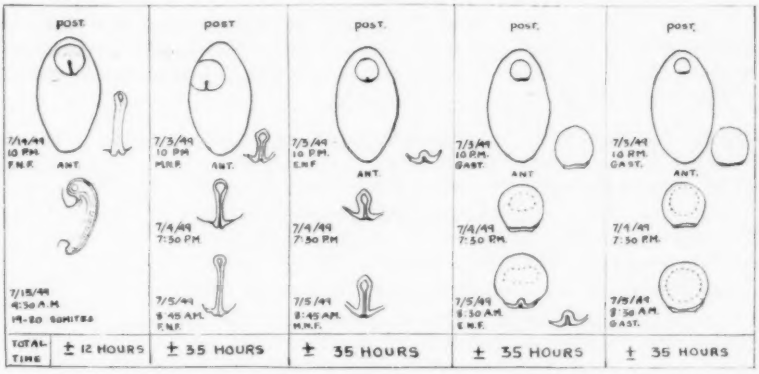

FIGURE 8. Rate of development of living embryos of *M. canis.* Labels indicate the orientation of ova in utero. Eggs in egg-cases were placed on wet cotton in covered finger bowls set in running sea water at 22–23° C.

Clark (1926), at the Plymouth Laboratory, opened egg capsules of *R. brachyura* tagged at the time of spawning and found cleavage stages through the fourth day of incubation. His next report is that of an early neural fold stage, aged 24 days, while embryos of 4 to 4.5 mm. were observed after 35 and 43 days. Clark's series is incomplete with no data for embryos between 7.5 and 58.5 mm. nor did he follow the development of individual eggs. The exceedingly slow rate in *R. brachyura* as compared with that of other elasmobranchs mentioned above may be a species difference and may be attributed in part to the low temperature of the laboratory tanks at Plymouth (13.4–16° C.).

On the basis of the above data on the development of individual eggs of *M. canis,* together with the marked difference in stage and arrangement in sequence of the embryos in a uterine series, it can be estimated that successive ovulations usually of two eggs, occur in this species about every 30 to 40 hours. This is a reasonable estimate in view of Clark's (1922) observation of a 24 hour interval between spawnings in *R. brachyura* but it is an approximation only. More data on living embryos are necessary before a definite statement can be made. Assuming, however, that *M. canis* ovulates about every 36 hours, it can be calculated that in the fish represented by Series A (Fig. 7, 7/3/49), the first ovulation occurred about June 22 and the last about July 1; in Series C (7/9/49), the first ovulation took place about June 20, the last about June 27; Series F (7/14/49), first, June 29; last,

[5] The capsule of the skate egg may be shaved thin over the embryo to facilitate observation.

July 9. In Series B (7/7/46) the great difference between the two youngest embryos suggests an interval of about 5 days between ovulations, an instance of irregularity in the cycle. From records of a number of series of embryos (Table I) it is concluded that ovulation in *M. canis* usually begins between June 20 and July 7 and is terminated between June 25 and July 20.

An ovulatory period limited to a few weeks in the summer is in distinct contrast to the situation in oviparous elasmobranchs, many of which have been found to breed throughout the year (Clark, 1922; Metten, 1939). In species with uterogestation, however, it is not surprising that ovulation is confined to a short period between pregnancies. That such is also the case in *Squalus acanthias* is known from the work of Hisaw and Albert (1947) who report that ovulation occurs every two years following a gestation of 20 to 22 months.

Egg-cases without yolk. Occasionally empty egg-cases are found in the uterus of *M. canis*. In one instance (8/4/46) a fish opened soon after collecting contained 5 eggs in cases in the right uterus and 5 cases with albumen but no sign of yolk in the left uterus. There were no crushed or disintegrating ova. Embryos in the right uterus measured 15 to 28 mm. in length and were in excellent condition. A possible explanation of this phenomenon is that the lumen of the left oviduct had been occluded (a point unfortunately not determined) so that all ova entered the right oviduct.[6] Since 5 empty cases were present in the left uterus, however, the conclusion seems warranted that at each ovulation of an egg, or eggs, secretion of albumen and egg-case by both right and left oviducal glands occurs. If only one egg is ovulated, one of the two cases contains no yolk.

This assumption is supported by two instances in which, when the uteri of a specimen contained an unequal number of ova, an empty egg-case was found in the uterus with the smaller number in a position corresponding to that of the last egg ovulated on the opposite side (7/3/49; 7/7/49). In some specimens where unequal numbers of eggs were found, it was probable that one or more eggs had been extruded from a uterus, but in the two examples given above, the stages of embryos present, the position of the empty egg-cases at the most anterior end of the uterus, and the absence of any trace of yolk eliminated such an explanation.[7]

In a few instances skate egg-capsules spawned in Dr. Hartmann's aquarium contained no yolk. Clark (1922) in his report on rays and skates, noted "a small percentage of perfectly formed, undamaged egg-capsules contained some albumen but no yolk."[8] He suggested that such capsules had been closed before eggs reached them but it seems entirely possible that such yolkless capsules may be other examples in which both oviducal glands secreted egg-cases although only one egg was ovulated.

Metten (1939) presents conclusive evidence in *Scylliorhinus* that the ovum does not act as a direct stimulus for the secretion of the egg-case, in showing that

[6] When this specimen was obtained, the probability of the ovulation of two eggs at one time was unknown to the author so that the size of each of the five embryos was not recorded. It is possible that there were pairs of embryos of the same size, but the range of 15 to 28 mm. suggests the unusual occurrence of five single ovulations at spaced intervals.

[7] In 5 specimens with unequal numbers of ova (Table I), no data were recorded on the presence or absence of empty egg-cases because at the time the writer was concerned chiefly with securing embryos for another study and did not realize the importance of such evidence.

[8] P. 580.

over one-half of the elaborate case of this species is formed before the egg enters the oviducal gland. He cited one instance also of a fully formed egg-case lacking the yolk.

Although experimental work is needed for definite proof, it is highly probable that oviducal glands in elasmobranchs, as in other vertebrates, are controlled by endocrine secretions. Wallace (1903) noted corpora luteal tissue in ruptured ovarian follicles of *Spinax,* and Hisaw and Albert (1947) have reported the differentiation of luteal tissue after ovulation in *Squalus acanthias.* It is not unlikely, therefore, that ovarian hormones, present at the time of ovulation or slightly preceding it, stimulate the secretion of a single egg-case by each oviducal gland irrespective of the number of ova discharged. This assumption would explain the occurrence of empty egg-capsules as well as that of "double-yolked" eggs.

Summary

1. The ovum and egg-case of *Mustelus canis,* a viviparous elasmobranch, are described and the orientation of the egg-case in utero is noted.

2. The blastoderm is typically located near the cloacal end of the yolk and the dorsal lip of the blastopore forms at the anterior border of the blastoderm when the egg is oriented as it lies in utero. Occasional cases of reversed and oblique orientation are found.

3. In early June, mature females are post-partum. The functional right ovary contains large ova soon to be ovulated while the left ovary is vestigial.

4. The ovulation period extends from about June 20 to July 20. Four to eight ova are generally found in each uterus of a pregnant female and, from a comparison of stages present, it is probable that as a rule two ova are discharged simultaneously with an interval tentatively estimated at 30 to 40 hours between most subsequent ovulations.

5. Ova are arranged in sequence in each uterus with the most advanced embryo nearest the cloacal end.

6. Stages of embryos present in utero between June 23 and Aug. 31 are tabulated. An egg ovulated at the beginning of this period would develop into an embryo with a maximum length of about 80 mm. in 9 weeks time.

7. From observations made on living material, blastoderms with thickened dorsal lips (gastrulation stages) are estimated as approximately 5 days younger than fused neural tube stages 3.5 to 4 mm. in length.

8. On the basis of egg-cases devoid of yolk as well as from the occurrence of "double-yolked" eggs, it is suggested that oviducal glands are controlled by ovarian hormones and that a single egg-case is secreted simultaneously by both right and left glands irrespective of the number of ova discharged at a given ovulation.

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THE COMPARATIVE EFFECTS OF TELEOST AND BEEF PITUITARY ON CHROMATOPHORES OF COLD-BLOODED VERTEBRATES[1]

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Treatment of most cold-blooded vertebrates, including catfish and eels, with pituitary gland induces a distal migration of pigment in their melanophores (Parker, 1948). However, there is evidence that the melanophores of some teleosts, such as killifish and Phoxinus, react oppositely to this treatment. In these fish the melanin concentrates (Hewer, 1926; Matthews, 1933). This variance with teleosts may be due to physiologically distinct chromatophores or it may be caused by differences in the source or preparation of the pituitary used. For instance, commercial preparations often contain such preservatives as chloretone which also effect color changes and therefore may lead to false interpretation.

An effort is made here to compare the effects of pituitary extracts from two widely separated groups of vertebrates, teleosts and mammals, on the chromatophores of elasmobranchs, teleosts, amphibians and reptiles to determine if there are differences in either the extracts or the target organs. Particular emphasis is placed on the chromatophores of the anomolous bony fishes.

Materials and Methods

The following animals were utilized for this study: the elasmobranch *Urobatis halleri* (round stingray); the teleosts *Ameiurus melas* (black bullhead), *Fundulus parvipinnis* (California killifish), *Gambusia affinis* (mosquito fish), *Leuresthes tenuis* (grunion), *Girella nigricans* (opaleye), *Lepomis cyanellus* (green sunfish), and *Gillichthys mirabilis* (mudsucker); the amphibians *Hyla regilla* (Pacific tree frog) and *Rana pipiens* (meadow frog); and the reptile *Anolis carolinensis* (Carolina anole). After being adapted to white and black backgrounds, these animals were treated with extracted whole pituitary from fish and beef. Fundulus and Girella were also injected with Parke, Davis and Co. obstetrical pituitrin and beef posterior lobe extracts. Controls, similarly adapted, were injected with fish brain or with saline solution, or remained untreated. The controls always had the same light and temperature as the test animals.

The fish pituitaries were from daily landings of sheepshead (*Pimelometopon pulchrum*), barracuda (*Sphyraena argentea*), yellowfin tuna (*Neothunnus macropterus*), and white sea bass (*Cynoscion nobilis*). The pituitaries from different species were not mixed, although no differences were observed in their effects. Most of the experiments and all the final tests were performed with barracuda preparations. The whole gland was used since it was found impractical to separate the

[1] This investigation was aided by a grant from the Research Committee, Montana State University. Most of the work was done at Scripps Institution of Oceanography, La Jolla, California and includes part of the material in a thesis submitted to the University of California at Los Angeles in partial fulfillment of the requirements for the Doctor of Philosophy degree.

FIG. 1

FIG. 2

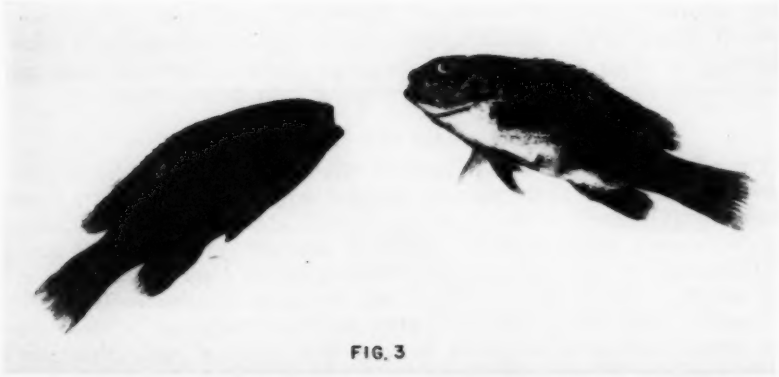

FIG. 3

FIG. 4

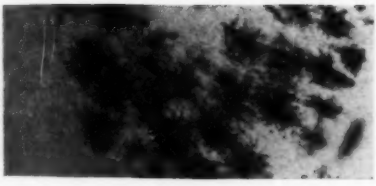

FIG. 5

FIGURE 1. Effect of fish pituitary on the coloration of *Urobatis halleri* kept on a white background. The dark ray on the left had been injected with fish pituitary and the pale ray on the right with fish brain. Photograph of living specimens.

FIGURE 2. Effect of fish and beef pituitary on the coloration of *Fundulus parvipinnis* kept on a black background. The two pale fish had been treated with fish pituitary and the two dark ones with beef pituitary. Photograph of live fish.

small, united lobes. The glands were placed in acetone and finely ground in a mortar. The powder was extracted five or six times with acetone, air dried, and put in glass ampoules which were sealed under reduced pressure. Over 1000 fish pituitaries were processed in this manner. One barracuda pituitary was equivalent to 4.2 mg. of the desiccated material.

Beef pituitaries were treated in the same fashion as the fish glands. Extracts were made of both the whole gland and the posterior lobe of freshly killed animals.

Injections were made intraperitoneally or, in the case of adult frogs, in the dorsal lymph sac. The dosages used in each test are recorded in the results.

Results

Urobatis halleri

Black-adapted and white-adapted rays were each injected with 7 mg. fish pituitary. Similarly adapted rays, serving as controls, were injected with 7 mg. fish brain. They were replaced in their respective white and black containers. After 2 hours those which had been treated with pituitary and were on a white background were slightly darker and had more distinct mottlings than those on the same background which had been treated with brain (Fig. 1). The darkening effect of the pituitary endured for 18 hours. No differences could be detected in the coloration of rays in black containers. Twenty-four hours after their treatment with brain, the controls were injected with fish pituitary. They darkened.

Ameiurus melas

Bullheads placed in dimly lighted aquaria for 48 hours paled to yellow ocher over the entire body, except on the tips of the barbels and fins. They became black when injected with either 5 mg. barracuda pituitary, 5 mg. whole beef pituitary or 0.2 cc. pituitrin, but remained pale when treated with fish brain.

Fundulus parvipinnis

Due to an obvious difference in coloration between mature males and females, only healthy female killifish were utilized.

Ten white-adapted and 10 black-adapted animals treated with 2 mg. fish pituitary became pale within 20 minutes (Fig. 2). They became slightly darker after 6 hours, but whether on a white or black background, they remained noticeably paler than any of the controls for 72 hours.

Beef whole gland was administered in a larger dose (3 mg.) than was the fish extract, yet it caused no change in the coloration of white-adapted or black-adapted fish (Fig. 2). The same was true for the fish treated with extracts of the posterior

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Figure 3. Effect of fish pituitary and pituitrin on the coloration of *Girella nigricans* kept on a black background. The pale fish on the right had been treated with pituitary and the dark one on the left with pituitrin. Notice the pale iris of the fish injected with the fish extract. Photograph of live fish.

Figure 4. Photomicrograph of a portion of a scale from Fundulus injected with fish pituitary. The melanin is punctate.

Figure 5. Photomicrograph of a portion of a scale from Fundulus injected with beef pituitary. The melanin is diffuse. Untreated controls appeared the same.

lobe of beef or with pituitrin. Controls injected with Ringer's solution or fish brain also exhibited no color change.

As many as 6 fresh Fundulus glands, implanted in the body cavity, did not pale black-adapted killifish. However, when a greater quantity of fresh fish material (2 glands of Sebastodes) was introduced, enough of the substance was present to cause them to blanch to a light yellow, even though they were on a background conducive for darkening. Killifish implanted with a comparable amount of fresh fish brain adapted to their black background.

The color of the variously treated fish was correlated with the microscopic appearance of the chromatophores (Table I). Fish pituitary, like adrenalin, caused a proximal migration of melain, whereas barracuda brain and mammalian extracts had no apparent effect on black- or white-adapted animals (Figs. 4 and 5).

TABLE I

Microscopic appearance of melanophores on scales of Fundulus parvipinnis

Material injected	Total number melanophores counted*	% Punctate	% Intermediate	% Diffuse
Barracuda brain	1211	3	16	81
Beef whole pituitary	894	9	22	69
Beef posterior lobe	1055	6	7	87
Obstetrical pituitrin	1107	2	13	85
Desiccated fish pituitary	1291	76	19	5
Fresh fish pituitary	988	68	19	13
Adrenalin chloride	1402	88	11	1

* Counts were made on scales from two fish for each material injected. Each circulus of a scale was taken in turn and followed around.

Punctate = melanin concentrated in a small spot in the center of the melanophore; Intermediate = stellate, melanin only partially dispersed; Diffuse = melanin well dispersed in the radiating processes of the melanophores.

The xanthophores of *F. parvipinnis* are much smaller than the melanophores and their radiating processes are difficult to discern. Pale fish, which had been treated with fish pituitary, showed most of the xanthophores expanded. In the dark fish the xanthophores were largely obscured by the radiating processes of the overlying melanophores. Accurate count of expanded or contracted xanthophores could not be made.

The fish extracts were also tested on Fundulus with denervated dark bands on their caudal fin. The method used in denervation was essentially the same as that employed by Parker (1934). A cut was made across two caudal fin rays just posterior to the basal blood vessel arc of the tail. Within a few hours a dark band of expanded melanophores was formed distal to the cut which, according to Parker, results from dispersing nerves stimulated by injury. Ten specimens with such denervated bands were injected with fish pituitary. The dark denervated bands paled like the rest of the body.

Girella nigricans

Ten olivaceous black opaleyes, injected with sufficient fresh fish pituitary or with 3 to 4 mg. fish pituitary extract, commenced to blanch in 10 to 30 minutes and

remained pale for 30 hours. The normally blue iris became gold for the first few hours after injection (Fig. 3).

Barracuda brain, pituitrin and extracts of beef whole gland or posterior lobe had no apparent action.

Any disturbance caused light-colored opaleyes, whether pale due to sojourn on a white background or from injections of teleost pituitary, to darken when handled or otherwise disturbed. Some microscopic analysis of color change, however, was possible throught a study of their scales. The extreme pallor of those injected with the teleost pituitary was confirmed as being due to concentration of melanin.

The outlines of the xanthophores of Girella are more clearly defined than are those of Fundulus. Eight-hundred to 1200 of these chromatophores were counted on scales from each fish. Xanthophores of fish treated with fish pituitary averaged 93 per cent expanded and 7 per cent intermediate, whereas for fish treated with mammalian preparations or fish brain they ranged from 8–28 per cent expanded. The majority were punctate.

Gambusia affinis, Leuresthes tenuis, Lepomis cyanellus, Gillichthys mirabilis

Mosquito fish, grunion, sunfish and mudsuckers all paled and remained so for at least 48 hours after treatment with fish pituitary. At least 10 fish of each species were tested. The mosquito fish became so transparent on the head that the outlines of the brain showed, indicating that the perineural as well as the skin melanophores had contracted. None of these species showed a visible reaction to fish brain or to mammalian preparations.

Hyla regilla

Six white-adapted frogs became practically black one hour after having 1 to 3 mg. fish pituitary injected into their dorsal lymph sac. Those which received the larger doses did not darken more quickly than those which received smaller amounts. However, there was a correlation between the dosage and the duration of melanophor expansion. Frogs injected with 3 mg. remained dark for approximately 96 hours, whereas those which received 1 mg. returned to the light phase in 18 hours. The effects of a 2 mg. dose lasted 24 hours.

Six controls treated with fish brain exhibited no color change.

Rana pipiens

Twenty white-adapted meadow frogs injected with either fish or beef pituitary darkened within an hour. Even the gold in the iris was supplanted by black. Some commenced to pale by 24 hours, and it was noticed at this time that the iris had largely regained its gold. Sections of frog skin in saline suspensions of beef or fish pituitary also darkened.

No differences were noticed between the action of the fish and beef preparations. No attempts were made to assay the materials, but when used in the same concentrations (6 mg.), the fish and beef extracts induced darkening at approximately the same time and the effects wore off at about the same time.

There were no observable color changes in any of the various controls nor in black-adapted frogs injected with pituitary preparations.

Tadpoles of Rana pipiens

Tadpoles which received either fish or beef pituitary and were then placed in white porcelain bowls became considerably darker than controls. Microscopic examination of the tails of these tadpoles revealed that the majority of the melanophores had diffuse melanin and that most of the leucophores had concentrated pigment (Table II). Many of the dermal melanophores formed a blotchy reticulum involving several of these black chromatophores. On the other hand, most of the melanin was concentrated and the guanine of the leucophores was dispersed in the tails of controls; a condition just reverse to that in animals treated with pituitary.

TABLE II

*Microscopic appearance of chromatophores on tails of Rana pipiens larvae**

Material injected	Melanophores			Leucophores		
	Punctate	Intermediate	Diffuse	Punctate	Intermediate	Diffuse
Barracuda brain	70	27	3	31	69	0
Ringer's	76	18	6	20	60	20
Barracuda pituitary	20	10	70	95	5	0
Beef pituitary	23	18	59	88	12	0

* Counts were made on two individuals for each different material injected. A total of 500 chromatophores of each type was counted in each individual. The counts are expressed as percentages.

Anolis carolinensis

It was found best to make observations on the lizard at night because, although the dark-adapted lizards were kept in battery jars painted black and covered to exclude light, they were more uniformly green at night than during daytime. Anolis exhibits a diurnal melanophore rhythm, which is attributable to the pituitary (Rahn and Rosendale, 1941).

Fifteen green, dark-adapted lizards injected with 4 mg. barracuda pituitary commenced to darken at the point of injection in approximately 1 minute. Twelve treated with the same amount of beef whole gland reacted similarly. In an hour their backs were black to chestnut brown, which color they retained, night as well as day, for 30 hours. Another group of 15 lizards which received 4 mg. barracuda brain or which were untreated remained green throughout the night, darkened slightly the next day, and were green again on the following night.

Compared with their controls, brown lizards from lighted jars showed the effects of the pituitary only at night. During the day the controls were about as dark as the experimentals.

Sections of skin from the backs of green lizards, placed in suspensions of fish or beef pituitary and Ringer's solution, also proved that both extracts had a darkening action.

DISCUSSION

Investigators agree that hypophysectomy causes elasmobranchs to blanch and, conversely, that they darken when injected with pituitary of their own species or with

the mammalian gland (Lundstrom and Bard, 1932; Hogben, 1936; Parker, 1936). My experiments indicate further the non-specific darkening effect of pituitary inasmuch as rays (*Urobatis halleri*) were shown to darken slightly when treated with fish pituitary.

Unlike the elasmobranchs, different species of bony fishes exhibit diverse reactions to the chromatic principle (or principles) of the pituitary. Removal of the pituitary of Ameiurus causes the melanin to concentrate (Osborn, 1941; Parker, 1941), but a similar operation on Fundulus apparently has little or no effect on the state of the melanophores (Matthews, 1933; Fries, 1943). Injections of either teleost, amphibian or mammalian pituitary causes Ameiurus to darken (Odiorne, 1933; Parker, 1941). On the other hand, Odiorne was unable to show any melanophore response with pituitrin treated Fundulus, and Matthews (1933) observed that the melanophores on isolated scales of the same species contracted and the xanthophores expanded when they were treated with fish pituitary.

My results with Fundulus injected with fish pituitary agree most closely with Matthews' work as cited above and with Hewer's (1926) observations that cod pituitary contracts melanophores but expands xanthophores of Phoxinus. The paling of my killifish was so striking that there could be no mistaking the reaction, and the contracted condition of the melanophores was fully verified by microscopic examination. Gambusia, Gillichthys, Lepomis, Leuresthes and Girella all reacted similarly to Fundulus. It is very probable, however, that other species of fish may react like catfish and eels. Certainly no generalized rule can now be made for the color changes that teleosts as a group undergo when treated with pituitary preparations.

The literature on the subject of teleost response to mammalian preparations likewise indicates that all fish do not respond in a similar manner. However, a wide variety of hormone preparations have been used, and it is doubtful if any two contained similar pituitary substances in equal concentration. Using Fundulus alone as an example, commercial posterior lobe extracts causes melanophores to contract (Wyman, 1924); obstetrical pituitrin has no effect except on denervated areas where it expands melanophores (Kleinholz, 1935); and pituitrin has no action on melanophores but antuitrin contracts them (Odiorne, 1933). Although the mammalian extracts used in this study obviously darkened Ameiurus, they had no visible effect on the other bony fishes tested.

With one exception, melanophores of amphibians expand when the animals are treated with teleost, amphibian, reptilian or mammalian pituitary (Allen, 1920; Kleinholz, 1935; Gray and Ford, 1940). The exception is Diemyctylus, which reacts oppositely by paling when injected with pituitrin (Collins and Adolph, 1926). My results with amphibians were like those of previous investigators in indicating no specificity of chromatophorotropic hormone.

Falling in with the main line of cold-blooded vertebrate evolution, the reptiles are also uniform in their general response. Hypophysectomy leads to a permanent pallor (Parker, 1938; Rahn, 1941). Injection of fish, amphibian, reptilian, avian, or mammalian pituitary darkens them (Kleinholz, 1938, 1940; Parker, 1938; Rahn, 1941). In this study, the lizards behaved as expected from reports by previous investigators. Both fish and beef pituitary darkened green Anolis. There did not appear to be any qualitative difference in reaction to fish or beef preparations.

It would appear, therefore, that melanophores of nearly all cold-blooded vertebrates except the bony fishes react similarly to the chromatophorotropic principle. Also, pituitaries from all classes of vertebrates apparently have a like effect on all but the teleosts. The diverse chromatic reaction of teleosts seems to have a phylogenetic significance. Species, such as Ameiurus and Anguilla, have melanophores which react like those of other cold-blooded vertebrates. Species belonging to the higher orders, which have diverged more from the common trunk of vertebrate evolution, such as Fundulus and Girella, have melanophores which react oppositely to fish pituitary and exhibit little or no reaction to mammalian preparations.

There is at present no reason to believe that melanophores in different species cannot react oppositely to the same stimulus. It has been shown that potassium chloride will expand amphibian melanophores (Thörner, 1929) but contract those of certain fish (Spaeth, 1913). Also, different types of chromatophores in an individual animal may react differently to the same stimulus as was demonstrated in this investigation with tadpoles, Fundulus and Girella and by Allen (1925), Hewer (1926) and Matthews (1933).

Not only is there a differential reaction of melanophores in different species, as well as of different chromatophore types in an individual, but pituitary extracts from different sources are seen to be unlike in their chromatophorotropic activity when tested on higher bony fishes. The chromatic changes induced in Fundulus and Girella by fish extracts were undeniably different from their negative response to mammalian extracts. The mammalian material contained a melanophore expanding principle which was effective on catfish, frogs and lizards but which was ineffective on chromatophores of advanced fish, presumably because of low sensitivity or non-sensitivity of the fish target organs to this substance. The teleost extracts have a principle which, although like the mammalian, is capable of darkening rays, catfish, frogs and lizards, and differs in that it contracts melanophores of higher teleosts.

It is possbile that there are two chromatophorotropic factors in the fish pituitary. This appears to be the case for amphibians (Hogben and Slome, 1936; Steggerda and Soderwall, 1939). If there are two factors in the fish pituitary, then the melanophores of higher fish must be more sensitive to the contracting one than to the expanding one, whereas the melanophores of elasmobranchs, etc. must be most sensitive to the expanding principle.

It has not been the purpose of this investigation to determine whether the pituitary plays a normal role in the color changes of fish. The dosages used were purposely held large in order to elicit responses that would be definite. Also, the animals were placed in an environment which would normally effect color changes opposite to that wrought by pituitary treatments, in order to make more evident the comparisons with controls. Large doses were required to overcome the opposition factors.

Summary

1. Fish and beef acetone-desiccated pituitaries cause a distal migration of pigment granules in melanophores when injected into the elasmobranch *Urobatis halleri*, the teleost *Ameiurus melas*, the amphibians *Hyla regilla* and *Rana pipiens*, and the reptile *Anolis carolinensis*. These preparations not only disperse melanin but also

induce a proximal migration of the white granules in leucophores of *Rana pipiens* larvae.

2. Fish pituitary injected into the teleosts *Gambusia affinis, Fundulus parvipinnis, Girella nigricans, Gillichthys mirabilis, Lepomis cyanellus* and *Leuresthes tenuis* causes a proximal migration of melanin and, at least in the first three species, a distal migration of pigment in the xanthophores. Desiccated fish brain, beef whole gland, beef posterior lobe and commercial pituitrin have no obvious effect on their chromatophores.

3. There is an opposite migration of melanin in certain different species of teleosts treated with fish pituitary. The diverse chromatic reaction of teleosts may have phylogenetic significance. The more primitive teleosts appear to react like other classes of cold-blooded vertebrates, whereas the higher teleosts, which have diverged more from the common line of vertebrate evolution, do not.

4. There is a differential in reaction of different types of chromatophores in a single species to treatment with the same pituitary preparations.

5. Pituitary material from different sources may have unlike chromatophorotropic action. Beef and fish pituitary have a similar action (expansion) on melanophores of Urobatis, Ameiurus, Rana and Anolis. However, on such teleosts as Fundulus and Girella, fish extracts have the opposite effect and mammalian extracts have no apparent effect.

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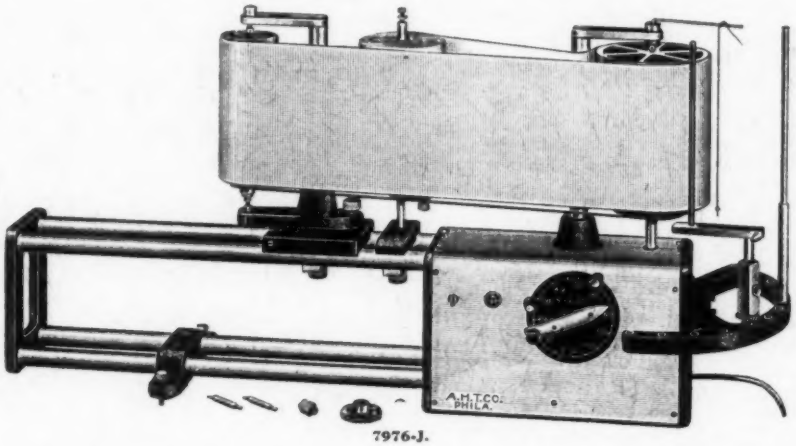

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